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(54) Title: SPORE-LIKE CELLS AND USES THEREOF

(57) Abstract: Highly undifferentiated spore-like cells can be isolated from many different tissues and bodily fluids and used to treat a wide variety of disorders.

SPORE-LIKE CELLS AND USES THEREOF

Field of the Invention

The invention relates to compositions and methods for tissue engineering and cell therapies.

Background of the Invention

The relatively new field of tissue engineering has provided alternatives to many previously tried methods for restoring tissue function. The field is an interdisciplinary one that applies, primarily, engineering and life science principles to develop biological substitutes that maintain, improve, or restore tissue function (*Tissue Engineering*, R. Skalak and C.F. Fox, Eds., Alan R. Liss, New York, NY, 1988; Nerem, *Ann. Biomed. Eng.* 19:529, 1991).

Tissue engineers have used three general strategies to create new tissue. The first strategy employs isolated cells or cell substitutes. This approach avoids many of the complications of surgery (*e.g.*, complications that arise following organ transplantation), allows replacement of only those cells that supply the needed function, and permits manipulation of cells before they are administered to a patient. However, the cells used do not always maintain their function in the recipient and can evoke an immune response that results in their destruction.

The second strategy employs tissue-inducing substances. For this approach to succeed, appropriate signal molecules, such as growth factors, must be purified and appropriately targeted to the affected tissue. While there is some understanding of how particular cells respond to particular growth factors, it is not currently possible to regulate, through extrinsic application of signaling molecules, the growth and differentiation of each cell type and to orchestrate the formation of three-dimensional organ structures.

The third strategy employs cells placed on or within matrices. In closed systems, these cells are isolated from the body by a membrane that is permeable to nutrients and wastes, but impermeable to harmful agents such as antibodies and immune cells. Closed systems can be implanted or used as extra-corporeal devices.

In contrast, in open systems, cell-containing matrices are implanted and become incorporated into the body. The matrices are fashioned from natural materials such as collagen or from synthetic polymers. The risk of immunological rejection is lessened by systemic administration of immunosuppressive drugs or by the use of autologous cells. Of course, immune suppression places the patient at substantial risk (*e.g.*, the patient is at risk of contracting infectious diseases), and it can be extremely difficult to obtain the requisite number of autologous cells.

Summary of the Invention

The present invention is based on the discovery of highly undifferentiated cells called spore-like cells, which can be isolated from many different tissues and bodily fluids and used to treat a wide variety of disorders. For example, spore-like cells can be used to reengineer damaged or diseased tissue, to augment existing tissue, to create new tissue, or to otherwise improve the condition of a patient who is suffering from a disorder that is amenable to treatment by a cell- or gene-based therapy.

Accordingly, the invention features an isolated spore-like cell that, when first isolated, is multipotent, less than one to approximately seven microns in diameter, and tolerant of oxygen deprivation. A spore-like cell is "first isolated" when it is separated from substantially all of the differentiated cells with which it is naturally associated in an organism for a period of time less than about four hours. Of course, spore-like cells can retain some or all of their unique features for longer periods of time depending on the culturing conditions, which can be selected to induce or inhibit differentiation and/or proliferation. For example, spore-like cells can remain tolerant of oxygen deprivation for more than four hours in culture. The cells of the invention can have one or more areas of high contrast when viewed by transmission electron microscopy (*i.e.*, areas of high contrast similar in appearance to the areas of contrast in the spore-like cell shown in Figs. 2C and 2D). Moreover, isolated spore-like cells can remain viable following exposure for more than ten minutes to an environment that is at least 42°C or less than 0°C. Cells that are tolerant of such extreme conditions can also have one or more areas of high contrast when viewed by transmission electron microscopy.

Any of the spore-like cells of the invention can be less than one (*e.g.*, one-tenth to one-third of a micron) to approximately seven (*e.g.*, one to seven or one to three) microns in diameter and isolated from a post-natal animal (*e.g.*, an adult animal such as an adult human). The cells can also be isolated from a deceased animal (*e.g.* a deceased human). Spore-like cells are widely distributed within these sources and can be isolated from a tissue that develops from the endoderm, mesoderm, or ectoderm. At least about half the volume of the cell can be comprised of nucleic acids and, when first isolated, the cell can fail to express the protein nestin when analyzed by immunocytochemistry. A spore-like cell fails to express the protein nestin when any apparent binding of an anti-nestin antibody to a spore-like cell is not significantly greater than that observed in a negatively controlled experiment. For example, a spore-like cell fails to express nestin when the signal generated by an anti-nestin antibody is not statistically significantly greater than the signal generated when no primary antibody is included in the reaction or when the primary antibody has been inactivated. Of course, the reaction must be carried out under conditions in which nestin would be bound if it were present. In other words, there must be an effective positive control. For example, the reaction must be carried out under conditions in which known nestin-positive cells are bound by an anti-nestin antibody.

Any of the spore-like cells of the invention, when isolated from a post-natal mammal and placed in a damaged, infected, or malfunctioning tissue, can develop into a cell having a phenotype substantially similar to the phenotype of a healthy cell normally found in the tissue. A spore-like cell develops into a cell that has a phenotype substantially similar to the phenotype of a healthy cell when the spore-like cell develops into a cell that expresses a protein that is deficient in the animal or tissue into which the spore-like cell is placed. For example, the phenotype is "substantially similar" when a cell that develops from a spore-like cell expresses alpha-galactosidase A, estrogen, a chloride channel, or insulin in an animal or tissue that is deficient in alpha-galactosidase A, estrogen, that chloride channel, or insulin. In the event tissue is damaged (*e.g.*, by trauma) or otherwise destroyed, the phenotype is "substantially similar" when a cell that develops from a spore-like cell expresses a protein once supplied by the damaged tissue. Given these examples, one of ordinary skill in the art can recognize many more "substantially similar" phenotypes. For

example, when isolated from the dermis of a post-natal mammal and placed in a dermal wound, a spore-like cell can develop into a cell having a phenotype substantially similar to that of a sympathetic or parasympathetic neuron, or a cell within a sweat gland, a sebaceous gland, or a hair follicle; when isolated from the epidermis of a post-natal mammal and placed in an epidermal wound, a spore-like cell can develop into a cell having a phenotype substantially similar to that of a melanocyte, a keratinocyte, or a Merkel cell; when isolated from the retina of a post-natal mammal and placed in a damaged or malfunctioning retina, a spore-like cell can develop into a cell having a phenotype substantially similar to that of a pigmented epithelial cell, a photoreceptor cell, a bipolar cell, a horizontal cell, an amacrine cell, a ganglion cell, an interplexiform cell, or a radial cell of Muller; when isolated from the pancreas of a post-natal mammal and placed in a damaged or malfunctioning pancreas, a spore-like cell can develop into a cell that produces glucagon, somatostatin, pancreatic polypeptide, or insulin; when isolated from the lung of a post-natal mammal and placed in a damaged or malfunctioning lung, a spore-like cell can develop into a cell that exchanges oxygen or secretes a surfactant.

The invention also features a tissue construct comprising a spore-like cell. A "tissue construct" is any medium, structure or device that contains or supports (physically or chemically) biological material, including material that consists of or includes the novel cells of the present invention. For example, a tissue engineering construct can include a biocompatible solution (*e.g.* a saline solution) that includes cytokines, growth factors, and antibiotics. The construct can include a support structure and, further, a hydrogel (the hydrogel and the spore-like cell form a hydrogel-spore-like cell composition).

In another aspect, the invention features methods for isolating a spore-like cell by dissociating a tissue sample and passing the dissociated tissue sample through a first device having an aperture no greater than about 15 microns. One can pass the tissue sample through a second device having an aperture greater than 15 microns before the sample is passed through the first device. One can also isolate spore-like cells by passing a tissue sample through a series of devices having progressively smaller apertures, the smallest aperture being about 15 microns. The first device, the second device, or one or more of the series of devices can be a pipette or filter. The tissue

can also be exposed to digestive enzymes, such as trypsin or collagenase. For example, the tissue can be incubated with approximately 0.05% trypsin at 37°C for approximately five minutes.

Spore-like cells and their progeny (*e.g.*, a skin, pancreatic, or retinal progenitor cell) must originally be isolated from their natural environment (*i.e.*, removed from a place where they reside within an animal) to fall within the present invention. Accordingly, an "isolated" spore-like cell or a tissue-specific (*e.g.* a skin, pancreatic, or retinal progenitor cell) can be a cell that is placed in cell culture, even temporarily. The term covers single, isolated spore-like cells and their progeny as well as cultures of spore-like cells that have been significantly enriched (*i.e.*, cultures in which less than about 10% of the cells are fully differentiated cells).

As used herein, the terms that are used to describe the progeny of a spore-like cell (*e.g.*, "progenitor" or "a spore-like cell and/or its progeny") refers to a descendent of a spore-like cell that differentiates into a cell having some but not all of the characteristics of a mature cell (*e.g.*, a pigmented epithelial cell, a photoreceptor (*i.e.*, a rod or a cone), a bipolar cell, a horizontal cell, an amacrine cell, a ganglion cell, an interplexiform cell, or a neuroglial cell (the radial cell of Müller)). Thus, the progeny of spore-like cells are distinguishable from the differentiated cell types naturally found in tissues such as the retina. Accordingly, cells are excluded from the invention when they assume characteristics that render them indistinguishable from previously identified stem cells (*e.g.*, mesenchymal stem cells), precursor cells (*e.g.*, the islet cell precursors described by Cornelius *et al.* (*Horm. Metab. Res.* 29:271-277 (1997)), or the progenitors from central nervous tissue described by Shihabuddin *et al.* (*Exp. Neurol.* 148:577-586 (1997)) or Weiss *et al.* (*J. Neurosci.* 16:7599-7609 (1996)) or terminally differentiated cells. These characteristics can be assessed by those of ordinary skill in the art in numerous ways (*e.g.*, by routine histological, biochemical, or, preferably, electron microscopic analysis).

A "hydrogel" is a substance formed when an organic polymer, which can be natural or synthetic, is set or solidified to create a three-dimensional open-lattice structure that entraps molecules of water or other solutions to form a gel. Solidification can occur by aggregation, coagulation, hydrophobic interactions, cross-linking, or similar means. Preferably, the hydrogels used in conjunction with spore-

like cells and their progeny solidify so rapidly that the majority of the cells are retained at the application site. This retention enhances new cell growth at the application site. The hydrogels are also biocompatible (*e.g.*, they are not toxic to cells). The "hydrogel-cell composition" referred to herein is a suspension that includes a hydrogel and a spore-like cell or its progeny.

The invention has many advantages. The compositions and methods described herein fill a therapeutic void. For example, there have been no successful attempts to repair damaged retinas by tissue engineering. Indeed, others have postulated that progenitor cells do not exist in the retinas of adult mammals (and no one has described cells having the characteristics of the spore-like cells described herein). For example, Reh and Levine have stated that, "[t]here is currently no evidence for a neural/glial stem cell at the ciliary margin in the adult mammalian retina, and the retina of the mature mammal does not show regenerative capacity after damage" (*J. Neurobiol.* 36:206-220 (1998), at 217). The compositions and methods of the invention can also be used to generate cells, for example, islet cells, that can not only be maintained in culture, but also expanded in culture to yield a large number of biologically active, for example, insulin-producing, cells. Thus, the new compositions and methods described herein make it possible to use a single tissue sample to produce enough cells to manufacture significant amounts of biological materials (*e.g.* proteins such as enzymes and hormones) and to replace missing, damaged, infected, or malfunctioning cells in a patient (*e.g.*, a diabetic patient). This is true even for spore-like cells that are precursors of cells that, when fully differentiated, are difficult to maintain or expand in culture (*e.g.*, neurons, hepatocytes, and islet cells). Other features and advantages of the invention will be apparent from the following detailed description and from the claims.

Unless otherwise defined, all technical and scientific terms used herein have the same meaning as commonly understood by one of ordinary skill in the art to which this invention belongs. Although methods and materials similar or equivalent to those described herein can be used in the practice or testing of the present invention, useful methods and materials are described below. All publications, patent applications, patents, and other references mentioned herein are incorporated by reference in their entirety. In case of conflicting subject matter, the present

specification, including definitions, will control. The materials, methods, and examples presented herein are illustrative only and not intended to be limiting.

Brief Description of the Drawings

Figs. 1A-1C are scanning electron micrographs of spore-like cells obtained from the liver of an adult rat. The cells are magnified 5,000X in Figs. 1A and 1B, and 10,000X in Fig. 1C. The scale bars represent 1.0 μ.

Figs. 2A-2D are transmission electron micrographs of spore-like cells obtained from the liver of an adult rat and placed in culture for 12 days. The magnification in Figs. 2A-2D is 25,000X, 39,000X, 17,000X, and 90,000X respectively.

Figs. 3A-3C are photographs of cells isolated from an adult rat heart and placed in culture. The newly isolated cells shown in Fig. 3A include undifferentiated spore-like cells (magnified 100X). After three days in culture, early myocardial cells can be seen (Fig. 3B). After two weeks in culture, Purkinje-like structures can be seen (Fig. 3C).

Figs. 4A-4C are photographs of cells isolated from the small intestine of an adult rat. The newly isolated cells shown in Fig. 4A include undifferentiated spore-like cells. After three days in culture, clusters of small intestinal cells (Fig. 4B) and autonomic neurons (Fig. 4C) can be seen. Figs. 4A-4C are shown at a magnification of 200X.

Figs. 5A and 5B are photographs of cells isolated from the bladder of an adult rat. The newly isolated cells shown in Fig. 5A include undifferentiated spore-like cells (magnification at 100X). After two days in culture, the isolated spore-like cells, or their progeny, appear to be differentiating (Fig. 5B; magnification at 200X).

Figs. 6A and 6B are photographs of cells isolated from the kidney of an adult rat. The newly isolated cells shown in Fig. 6A include undifferentiated spore-like cells (magnification at 100X). After three days in culture, aggregates of cells resembling kidney structures can be (Fig. 6B; magnification at 200X).

Figs. 7A-7E are photographs of cells isolated from the liver of an adult rat. The newly isolated cells shown in Figs. 7A and 7C include undifferentiated spore-like cells (magnification at 100X). After three days in culture, an aggregate of cells

resembling a differentiating liver structure can be seen (Fig. 7B; magnification at 200X). After seven days in culture, cells resembling hepatocytes can be seen (Fig. 7D). After 12 days in culture, many cells isolated from the liver express bile, as evidenced by a Hall's stain (Fig. 7E; 400X).

Figs. 8A-8C are photographs of cells isolated from the lung of an adult rat; Fig. 8D is a photograph of cells in a culture initiated by spore-like cells obtained from an adult sheep lung; and Fig. 8E is a photograph of a semi-thin section of a feline lung. The newly isolated cells shown in Fig. 8A include undifferentiated spore-like cells. After six weeks in culture, alveolar-like cells can be seen (Figs. 8B and 8C). After 30 days in culture, spore-like cells have formed alveolar-like structures (Fig. 8D) similar to those seen in the lungs of adult mammals (Fig. 8E).

Figs. 9A-9D are photographs of cells isolated from the adrenal gland of an adult rat. Undifferentiated spore-like cells can be seen at Day 0 (see the arrows in Figs. 9A (200X) and 9B (400X)). After two days in culture, primitive adrenal cells can be seen (Figs. 9C (200X) and 9D (400X)).

Figs. 10A-10C are photographs of islet-like structures. These structures formed in cultures of spore-like cells that were isolated from pancreatic tissue that contained no islets (the islets were harvested prior to the isolation of spore-like cells). After six days in culture, more than 100 islet-like structures were present per field (at 100X magnification; Figs. 10A and 10B). The islet-like structures were immunostained, which revealed insulin expression (Fig. 10C).

Fig. 11 is a photograph of a culture that includes undifferentiated spore-like cells isolated from adult human blood.

Figs. 12A and 12B are photographs of cultured cells. The cultures were established seven days earlier and contained spore-like cells isolated from adult human blood. In Fig. 12A, the cells are viewed with phase contrast microscopy. In Fig. 12B, the cells are illuminated with fluorescent light following immunohistochemistry for nestin.

Fig. 13 is a schematic of a permeable support structure filled with a hydrogel-spore-like cell composition.

Detailed Description

The present invention provides compositions and methods for repairing, replacing, or generating tissue (which can include a single cell type or a combination of cell types) or another biologically useful substance (*e.g.*, a hormone, an enzyme, or an anti-angiogenic factor). The compositions include spore-like cells (*e.g.*, mammalian spore-like cells) and certain of their progeny, either or both of which can be administered to a patient by the methods described below or by way of existing tissue engineering or cell therapy procedures known to those of ordinary skill in the art.

Spore-like cells, their novel progeny, and exemplary methods for their isolation and use are described below.

A. Spore-like Cells

1. Source

Spore-like cells and their progeny can be obtained from a donor (*e.g.*, a member of an avian, reptilian, amphibian, or mammalian class). For example, mammalian spore-like cells can be isolated from a rodent, a rabbit, a cow, a pig, a horse, a goat, a sheep, a dog, a cat, a non-human primate, or a human. Spore-like cells can be isolated not only from many different types of animals, but also during many different stages of the animal's life, including stages where the animals are quite mature (*e.g.* adolescence and adulthood). Notably, because spore-like cells tolerate oxygen deprivation and exposure to extreme temperatures better than differentiated cells (this ability is discussed further below), viable spore-like cells can also be isolated from deceased animals, including animals that have been deceased for many days, if not weeks, months, or years (*e.g.*, animals that have been deceased for 1,000 years or more).

In addition, spore-like cells can be obtained from a variety of sources within a given donor. For example, spore-like cells can be obtained from bodily fluids (*e.g.*, blood, saliva, or urine), and most, if not all, functional organs. While spore-like cells have been isolated from body fluids and solid functional organs, it is not clear whether they originate exclusively in either of these places. It may be that tissues and organs are the primary sources for spore-like cells, which appear in body

fluids only secondarily, for example, when the cells are "washed out" of those tissues. However, it is also possible that spore-like cells originate in bodily fluids or from the same source as other cells that are present in bodily fluids (*e.g.*, spore-like cells may originate only in the bone marrow). If so, spore-like cells could then be subsequently delivered from those fluids to specific tissues. Moreover, delivery can be upregulated when the tissue is affected by, for example, a disorder, a regenerative process, or wound healing.

The Examples below demonstrate that spore-like cells can be isolated from, *inter alia*, adult mammalian liver, lung, heart, bladder, kidney, and intestine, and can differentiate into hepatocytes, alveolar cells, cardiac myocytes, bladder cells, renal cells, and autonomic neurons, respectively. Given the variety of known sources for spore-like cells, it is reasonable to expect that these cells can be found in most, if not all, tissues and bodily fluids.

The donor of spore-like cells can be the recipient (*e.g.*, a human patient or an animal) who will be subsequently treated with those cells, another person, or an animal of, *e.g.*, the same or a different species from the recipient. In other words, autologous, allogenic, and xenogeneic spore-like cells can be obtained and used to treat human patients (methods of treatment are described further below).

Regardless of the source from which they are obtained, spore-like cells can be placed in culture, and cell lines derived from spore-like cells can be developed using techniques routinely practiced by those of ordinary skill in the art. Thus, cultured spore-like cells and cell lines derived from spore-like cells can also be used to treat human patients and are within the scope of the present invention.

2. Features and Characteristics

Spore-like cells were so-named because they have characteristics reminiscent of those of spores. Structurally, they have a primitive appearance and, functionally, they tolerate extreme conditions. Spore-like cells are typically small and generally spherical. Many cells in a culture of newly isolated spore-like cells are approximately 1 to 3 μ in diameter. Most spore-like cells have a diameter of approximately one to seven microns (*e.g.*, a diameter of one to two, two to four, three to five, about five, or five to ten microns). However, larger and smaller spore-like cells have been identified (*e.g.*, using electron microscopy). Given that spore-like

cells can differentiate into a variety of mature cell types, and that differentiation is a gradual process, it is difficult to define the precise upper size limit of spore-like cells. However, spore-like cells 4 to 5, and 7 to 10 μ in diameter have been identified in scanning electron micrographs. Occasionally, even larger cells (*e.g.*, cells as large as 12 to 18 μ) have been observed. From the observations to date, it is difficult to determine with certainty whether the apparent large cells are in fact single cells (perhaps on the verge of cell division) or conglomerates of several spore-like cells. The lower size limit of the spore-like cells is more definite and is certainly unique. Spore-like cells that are only about one-third of a micron in diameter (*e.g.*, one-tenth, one-third, one-fourth, one-fifth, or one-half micron) have been observed in scanning electron micrographs and some cells are as small as about one-tenth of a micron.

This extremely small size is in keeping with the unique composition of spore-like cells, which contain a great deal of nuclear material and relatively little cytoplasm. In most differentiated cells, the nucleus comprises approximately 10-20% of the cells' volume. However, approximately 50% and up to approximately 90% of the volume of a spore-like cell is comprised of nuclear material. Without limiting the invention to cells that arise by any particular mechanism, spore-like cells may arise when essential DNA fragments (which represent compressed DNA) are shed from mature cells (*e.g.*, those undergoing cell death by apoptosis or other means) and re-packaged and protected within, for example, a glycolipid-rich coat. Indeed, the concept of a minimal genome is beginning to emerge. This concept is exemplified by a mycoplasm that contains 517 genes but only requires 265 to 350 of these genes to survive (Hutchison *et al. Science* 286:2165-2169, 1999). If one considers the exquisite simplicity of DNA and the genetic code, it seems plausible that the complex information stored in DNA could be compressed considerably. The unique size of newly-isolated spore-like cell is perhaps best appreciated by viewing the cells with an electron microscope (*e.g.*, see Figures 1A-1C and 2A-2D).

The nuclear material appears to be surrounded by a coat containing more glycolipids and mucopolysaccharides than are normally found on the surface of normal differentiated cells. There are a number of standard assays for glycolipids, which are carbohydrate and lipid compounds that contain 1 mole each of a fatty acid, sphingosine, and hexose. Common reactions for carbohydrates include the periodic

acid-Schiff (PAS) reaction, diastase, alcian blue staining, colloidal iron, and hyaluronidase. Spore-like cells isolated from adult liver are stained by PAS and mucicarmine stains, which indicates that these cells are coated with mucopolysaccharids and glycolipids. In any event, the content of the coat is such that when the cells are viewed with a transmission electron microscope, they can appear to have a striped appearance (*i.e.* a plurality of areas of high contrast as shown in Figs. 2C and 2D). When the cells are exceedingly small (*e.g.*, less than about one micron) the areas of contrast are not as obvious, but they can nevertheless be seen with a trained eye.

Functionally, spore-like cells are unique in at least three ways. First, even though they can be isolated from a mature (*e.g.*, a post-natal, juvenile, adolescent, or adult) animal, they can differentiate into a variety of different cell types (*i.e.*, they are multipotent). For example, spore-like cells isolated from the lung develop not only into cells that mediate gas exchange, but also into cells that form the connective tissue and vasculature of the lung. Similarly, spore-like cells isolated from the liver develop not only into hepatocytes, but also into cells that form the connective tissue and vasculature of the liver. Thus, multipotent spore-like cells are those that produce some or all of the cell types found within complex tissue structures. At a minimum, spore-like cells must be shown to differentiate into two or more (*e.g.*, 2, 3, 4, or 5) cell types.

Second, spore-like cells tolerate exposure to extreme conditions (*e.g.*, dessication, oxygen-deprivation, and exposure to temperatures that are both much higher and much lower than normal body temperature (which, for warm-blooded mammals, is about 37°C)). As shown in the Examples below, spore-like cells can survive in low-oxygen environments, such as those that exist within the tissues of a deceased animal, or within a capped container of phosphate-buffered saline (PBS), for many hours (*e.g.*, four, six, ten, twelve, or 24 hours or more). While differentiated cells are able to survive oxygen deprivation for variable periods of time (*e.g.*, neurons are particularly sensitive, surviving only about 4-15 minutes after oxygen deprivation, while cartilage can remain viable, with refrigeration, for a month or so), spore-like cells isolated from differentiated tissue outlive the differentiated cells of that tissue when the tissue is oxygen-deprived. Moreover, spore-like cells can survive without

special preservatives. For example, hepatocytes can survive *ex vivo* for approximately two days if they are specially preserved, but spore-like cells isolated from the liver can survive for the same period (and much longer) without special preservatives.

Spore-like cells can also survive exposure to temperatures that are much higher and much lower than body temperature. For example, spore-like cells remain viable within tissues that are stored at about 4°C for a prolonged period of time (*e.g.*, one, three, five, seven, or more days). They also remain viable at temperatures that vary even further from a physiological body temperature. For example, substantially pure populations of spore-like cells (*e.g.*, spore-like cells isolated from a mammal) and spore-like cells within tissues (*e.g.*, spore-like cells within mammalian tissues) can survive freezing or heating to more than 5°C in excess of a physiological body temperature. That is, viable spore-like cells survive within tissues that have been cooled to 0°C, or below, or heated to about 43°C or above. As with oxygen-deprivation, spore-like cells can survive exposure to these conditions without special treatment (*e.g.*, they can survive exposure to freezing temperatures even without treatment with a cryopreservative). Viable spore-like cells can also be isolated from tissues that have been thoroughly dried (*e.g.*, by placement in a dessicator for approximately 24 hours). Given their ability to survive exposure to extreme conditions, spore-like cells can be isolated from an animal (including a human) that has been dead for many hours, for several days or weeks, or even longer. While most differentiated cells, particularly those within oxygen-sensitive tissues such as the brain, will no longer be viable, spore-like cells will be. Thus, the methods of the present invention are useful in forensic science to, for example, identify a deceased person. Similarly, spore-like cells can be isolated from tissues found at a crime scene, such as blood, and used to identify the perpetrator. Further, since spore-like cells can be isolated from tissues that have been frozen without a cryopreservative, they can be isolated from animals that have died in the wild in frigid climates and, quite probably, from animals that have been frozen for many, many years. Similarly, because spore-like cells remain viable even after exposure to heat, they can also be recovered from animals that have died in fires, arid landscapes, or in warm springs. Some of the animals from which spore-like cells can be isolated may now be extinct.

Third, under the conditions described in the examples below, spore-like cells have a greater capacity to proliferate than terminally differentiated cells isolated from specialized tissues such as the islet cells of the pancreas. When cultured under the conditions described in the Examples below, skin progenitor cells appear to double approximately every 24-36 hours. Cell viability and proliferative capacity can be assessed using standard techniques, including visual observation with a light or scanning electron microscope and Trypan blue exclusion. Proliferative capacity is an important attribute because tissue engineering, cell therapies, and gene-based therapies are often hampered by physicians' inability to obtain sufficient numbers of cells to administer to a patient.

The features and characteristics described herein can be used to distinguish spore-like cells from previously identified cell types. For example, the spore-like cells of the invention can be identified by one or more of the following attributes: their ability to differentiate into a variety of terminally differentiated cell types found in mature animals (such as those illustrated in the Examples below) and thereby give rise to complex tissue structures; their typical spherical shape, small size (as small as 0.1-0.3 μ in diameter and generally 1.0 to 3.0 μ in diameter), and cytoarchitecture (which includes relatively large amounts of nuclear material and relatively small amounts of cytoplasm); and their ability to survive in environments having a low or even non-existent oxygen supply.

3. Methods of Obtaining Spore-like Cells

To obtain spore-like cells, a sample of biological material is harvested from an animal (*e.g.* a human). Spore-like cells and their progeny can be obtained from the sample either immediately after collection (or soon after) or after the sample has been stored under either normal cell storage conditions or in an oxygen-poor environment or at a non-physiological temperature, for example, a temperature that varies from a normal body temperature by more than 5°C (*e.g.*, a temperature more than 42°C or at or below freezing). One of the easiest samples to obtain from an animal is a sample of whole blood. Those of ordinary skill in the art will appreciate that the isolation method will vary slightly depending on the type of tissue used as the starting material. For example, in the event the sample is a blood sample, it can be placed in a tube containing an anti-coagulant. After collection, tissue samples,

whether they are samples of bodily fluids or cell suspensions obtained from solid organs, are centrifuged for a time and at a speed sufficient to pellet the cells within the sample at the bottom of the centrifuge tube. The resulting pellet is resuspended in a suitable medium (*e.g.*, DMEM/F-12 medium supplemented with glucose, transferrin, insulin, putricine, selenium, progesterone, epidermal growth factor (EGF) and basic fibroblast growth factor (bFGF; see the Examples, below).

The suspended cells are then transferred to a tissue culture vessel and incubated at or near 37°C. Initially, when the sample is a blood sample, the culture flasks contain primarily hematopoietic cells. However, after several days in culture, the red blood cells lyse and degenerate so that the culture contains primarily, if not exclusively, spore-like cells. When spore-like cells are isolated from solid tissues, the differentiated cells can be lysed by triturating the sample with a series of pipettes, each having a smaller bore diameter than the one before. For example, the last pipette used can have a bore diameter of approximately 15 μ. After several additional days in culture, the spore-like cells multiply and can coalesce to form clusters of cells. Over time, usually on the order of approximately 7 days, their number can increase greatly. Typically, more than 90% of the cells are viable according to Trypan blue exclusion studies when isolated as described above.

When spore-like cells are obtained from a solid organ, for example by an excisional biopsy of the skin, the skin is swabbed with betadine and infiltrated with lidocaine. Under sterile conditions, a piece of skin can then be removed with a scalpel or punch. Once obtained, the dermis, the epidermis, or full-thickness skin can be placed in culture. If desired, spore-like cells or skin progenitor cells can be isolated individually from both the dermis and epidermis.

To obtain spore-like cells or skin progenitor cells, the piece of skin is placed in a buffered solution (*e.g.*, phosphate buffered saline), which can include one or more antibiotics, and the tissue can be dissociated mechanically (*e.g.*, by macerating the tissue or by scraping it with a scalpel or similar instrument), chemically (*e.g.*, by exposure to one or more enzymes, such as trypsin or collagenase, that facilitate tissue degradation), or both. Generally, the more aggressive the dissociation, the more spore-like cells or skin progenitor cells one will obtain. To isolate individual spore-like cells or skin progenitor cells, the tissue can be triturated,

first with a normal bore Pasteur pipette and subsequently with a series of fire polished pipettes having bore sizes ultimately reduced to less than about fifteen microns. This procedure (which is described in further detail in the Examples below) destroys large mature cells, but allows the smaller spore-like cells and skin progenitor cells (*i.e.*, cells generally having a diameter smaller than that of the smallest bore pipette) to survive.

The surviving cells are placed in an incubator and can be grown under conditions that either allow them to differentiate into the specialized cell types found in mature skin or that discourage differentiation. For example, spore-like cells and skin progenitor cells can be encouraged to differentiate by exposing them to the processes and basal nutrient media described in U.S. Patent No. 5,292,655. Alternatively, growth factors that cause progenitor cells to mitose (*e.g.*, epidermal growth factor (EGF), basic fibroblast growth factor (bFGF) and other cytokines) can be applied to help maintain the cells in an undifferentiated state. For example, the isolated cells can be cultured in Dulbecco's Modified Eagle's Medium (DMEM) supplemented with a hormone mixture containing glucose, transferrin, insulin, putricine, selenium, progesterone, EGF, and bFGF (see the Examples below). The media can be changed approximately every three days, and cells can be passaged approximately every 7-9 days.

Another way to promote proliferation is to expose spore-like cells or skin progenitor cells to agonists of Notch function, as described in U.S. Patent No. 5,780,300. Agonists of Notch include, but are not limited to, proteins such as Delta or Serrate or Jagged (Lindsell *et al.*, *Cell* 80:909-917, 1995) or biologically active fragments thereof. These proteins or protein fragments mediate binding to Notch and thereby activate the Notch pathway. Spore-like cells or skin progenitor cells can be contacted in culture with agonists of Notch or can be transfected with genes that encode Notch agonists. Techniques for transfecting cells in culture are routinely practiced by those of ordinary skill in the art. Progenitors that remain undifferentiated in culture can differentiate when administered to a patient; their differentiation being orchestrated by the microenvironment they encounter within the patient (differentiation is discussed further below).

Those of ordinary skill in the art will recognize that trituration through reduced bore pipettes is not the only way to isolate spore-like cells from larger, differentiated cells. For example, a suspension containing spore-like cells and differentiated cells can be passed through a filter having pores of a particular size. The size of the pores within the filter (and, similarly, the diameter of the pipette used for trituration) can be varied, depending on how stringent one wishes the isolation procedure to be. Generally, the smaller the pores within the filter, or the smaller the diameter of the pipette used for trituration, the fewer the number of differentiated cells that will survive the isolation procedure.

4. Spore-like Cell Differentiation

When first isolated, spore-like cells may not express any known markers (*i.e.*, proteins or other biological molecules associated with a given cell type, *e.g.*, a terminally differentiated cell type). After being placed in culture, some spore-like cells express nestin, a marker of neuroectodermally-derived cells. Those that do express nestin probably do so before expressing markers associated with terminally differentiated neural cells.

Spore-like cells or their progeny, when cultured, will eventually express cellular markers associated with terminally differentiated cells (see the Examples below), or can be made to do so by transfection with a gene encoding the marker or a biologically active substance of interest. Those of ordinary skill in the art can identify, by techniques routinely practiced in the art (*e.g.*, immunochemistry), numerous markers associated with terminally differentiated cells. For the purpose of defining the cells of the present invention, these cells become terminally differentiated (and thus fall outside the scope of the invention) when they express essentially the same phenotype as a mature cell. More specifically, a cell of the present invention is terminally differentiated when it expresses at least half of the distinguishing markers presently known in the art to be expressed by a particular mature (*i.e.* fully differentiated) cell. Antibodies to these markers are commercially available or otherwise readily attainable.

Alternatively, histological stains and microscopy can be used to identify mature cells. For example, electron microscopy can be performed to reveal melanosomes, and a Fontana stain can be performed to identify melanin granules in

melanocytes. Electron microscopy is also useful in identifying dense core granules in another epithelial cell type, the Merkel cell. Analysis of cellular morphology can also be used to identify sweat glands, which contain clear cells filled with glycogen, and sebaceous glands that are filled with lipids, triglycerides, cholesterol, and wax-like substances. To distinguish differentiated cells within the visual system, one can identify pigmented epithelial cells by the characteristic granules visible under the electron microscope; horizontal type I cells by the formation of connections with rods; horizontal type II cells by the formation of connections with cones; bipolar cells by their bipolar morphology and connections to rods or cones (they also send an axon to synapse with ganglion cells); amacrine cells by their numerous dendrites, but lack of an axon; interplexiform cells by their synapses with amacrine cells (they are pre- and post-synaptic to amacrine cells) and pre-synaptic connection with horizontal cells and bipolar cells; and ganglion cells as the terminal link in the neural network of the retina via their connection with the optic nerve.

One method of inducing differentiation is to allow spore-like cells or their progeny to establish contact (*e.g.*, physical contact) with a solid support. For example, spore-like cells can differentiate when they establish contact with a glass or plastic surface, a mesh, or other substrate suitable for use in tissue culture or administration to a patient. Contact can be facilitated by coating the solid support with one or more components of the extracellular matrix, such as laminin or fibronectin. On the other hand, keeping the cells suspended tends to inhibit differentiation.

Spore-like cells can also differentiate when they establish contact with a tissue within a patient's body or when they are sufficiently close to a tissue to be influenced by substances (*e.g.*, growth factors, enzymes, or hormones) released from the tissue. In other words, a spore-like cell can establish contact with a tissue (*e.g.*, the dermis or epidermis) by virtue of receiving signals from the tissue. Such signalling would occur, for example, when a receptor on the surface of a spore-like cell, or on the surface of a cell descended from a spore-like cell (*e.g.*, a skin progenitor cell), binds and transduces a signal from a molecule such as a growth factor, enzyme, or hormone that was released by a tissue within the patient.

Alternatively, or in addition, spore-like cells and their progeny can be induced to differentiate by adding a substance (*e.g.*, a growth factor, enzyme, hormone, or other signalling molecule) to the cell's environment. One of ordinary skill in the art can readily identify substances known to direct differentiation along various paths. For example, transforming growth factor alpha (TGFα) is known to stimulate amacrine cell differentiation (*J. Neurobiol.* 36:206-220, 1998) and to inhibit differentiation into rods (*J. Neurobiol.* 36:206-220, 1998). In contrast, retinoic acid stimulates rod differentiation and inhibits amacrine cell differentiation (*J. Neurobiol.* 36:206-220, 1998).

One or more substances that evoke differentiation can be added to a culture dish containing spore-like cells, to a mesh or other substrate suitable for applying spore-like cells to a tissue, or to a tissue within a patient's body. When a substance that induces spore-like cells to differentiate is administered, either systemically or locally, it can be administered according to pharmaceutically accepted methods. For example, proteins, polypeptides, or oligonucleotides can be administered in a physiologically compatible buffer, with or without a carrier or excipient. Of course, either the cells within a patient's body or the cells being administered (here, spore-like cells or their progeny) can be made to express particular factors following genetic manipulation. Thus, spore-like cells or their progeny can differentiate either in culture or in a patient's body, and can do so following contact with a solid support or exposure to substances that are either naturally expressed, exogenously administered, or expressed as a result of genetic manipulation. Regardless of the stimulus for differentiation, spore-like cells or their progeny are useful so long as they will differentiate or have differentiated sufficiently to aid in the maintenance or repair of an injured or malfunctioning tissue. For example, cells that have differentiated sufficiently to repair the dermis or epidermis can be administered to a patient at the site of a burn or other traumatized area of skin.

While spore-like cells or their progeny may eventually become fully differentiated, and while this is desirable in some circumstances (*e.g.*, where the cells are used to recreate a histologically mature and complete tissue), not all of the cells administered need to be fully differentiated to achieve successful treatment; spore-like cells or their progeny need only differentiate to a point sufficient to treat the patient.

That point can be reached either before or after the cells are administered to the patient. As described herein, the spore-like cell can also be genetically modified using routine techniques to express a beneficial enzyme or hormone (*e.g.* insulin).

B. Use

In view of the preceeding discussion and given the number of differentiated phenotypes already observed (see the Examples, below), it is reasonable to expect that spore-like cells can fully or partially differentiate into most, if not all, types of cells. Thus, spore-like cells can be used to study fundamental aspects of cellular differentiation. The rate and course of spore-like cell differentiation is influenced by the number and type of mature cells to which the spore-like cells are exposed. For example, when isolating spore-like cells from the liver, the more mature hepatocytes that remain in the culture of spore-like cells, the more quickly the spore-like cells will differentiate and the more likely it is that they will differentiate into hepatocytes. Thus, spore-like cells proliferate and differentiate in response to agents (*e.g.*, growth factors or hormones) within tissue, including tissue that has been injured or that is otherwise malfunctioning due to a medical condition, disorder, or disease. Regardless of their origin -- blood, another body fluid, the bone marrow, or a solid, functional tissue or organ -- spore-like cells can be influenced by agents that are either exogenously applied or provided by naturally occurring cells to which they have been exposed.

Spore-like cells can be used to maintain the integrity and function of a wide variety of tissues as well as to reengineer, repair, or otherwise improve tissue associated with a medical disorder. For example, spore-like cells can be used to maintain or reengineer tissue that develops from the endoderm, mesoderm, or ectoderm. More specifically, spore-like cells can be used to maintain or reengineer: bone; bone marrow; muscle (*e.g.*, smooth, skeletal, or cardiac muscle); connective tissue (*e.g.*, cartilage, ligaments, tendons, pleura, or fibrous tissues); lung tissue; vascular tissue; nervous tissue (*e.g.*, neurons and glial cells in the central or peripheral nervous systems), glandular tissue (*e.g.*, tissue of the thyroid gland, adrenal gland, or sweat or sebaceous glands); epithelial cells, keratinocytes, or other components of the skin; lymph nodes; the immune system; reproductive organs; or any of the internal

organs (*e.g.*, liver, kidney, pancreas, stomach, bladder, or any portion of the alimentary canal).

The preceeding list is intended to illustrate, not limit, the types of cells and tissues that can benefit from administration of spore-like cells (administration of spore-like cells is described further below). Those of ordinary skill in the art, given the present disclosure, will understand the wide variety of uses for spore-like cells. For example, life-like artificial skin can be produced by culturing spore-like cells and allowing them, when applied to a living body or used in conjunction with present skin replacement methods, to differentiate into epidermal and dermal cells (including melanocytes) as well as into hair follicles, sweat glands, sebaceous glands, ganglia, and similar adnexal structures.

Spore-like cells can also be used to treat diseases and disorders that affect other sensory systems. For example, spore-like cells can be used to treat blindness caused by, for example, retinal detachment. Presently, without treatment, retinal detachment often becomes total within six months (*see, e.g.*, Chapter 7 of *Current Medical Diagnosis and Treatment*, 37th Edition, Tierney *et al.*, Eds., 1998). Blindness can also result when the central retinal vein or its branches become occluded, as occurs in Behcet's syndrome, and visual acuity is impaired by systemic diseases such as diabetes mellitus, essential hypertension, preeclampsia-eclampsia of pregnancy, blood dyscrasias, and AIDS. Spore-like cells and retinal progenitor cells can be used to repair the retina when it has been injured or when its ability to function is otherwise compromised (*e.g.*, by macular degeneration, retinitis pigmentosa, retinal detachment, occlusion of a retinal artery or vein (or occlusion of one or more of their branches), or a retinopathy such as diabetic retinopathy, hypertensive retinochoroidopathy, blood dyscrasias, or cytomegalovirus retinitis). Spore-like cells or retinal progenitors, when administered to a patient, will differentiate into cells having some or all of the beneficial features of the following cell types: pigmented epithelial cells, photoreceptors (*i.e.*, rods or cones), bipolar cells, horizontal cells, amacrine cells, ganglion cells, interplexiform cells, and neuroglial cells (the radial cells of Müller). The cells of the invention can be placed in an area of the eye previously occupied by the retina (*i.e.*, the cells can be administered to a patient whose retina has been wholly or partially removed as a consequence of surgery or a disease process).

Spore-like cells and their progeny can also be used to treat patients who have a metabolic, enzymatic, or hormonal deficiency. For example, the compositions and methods of the invention can be used to treat a patient who has either Type I or Type II diabetes, who has suffered a traumatic injury to the pancreas, or who required surgery that adversely affected the pancreas (*e.g.*, surgical removal of some or all of the pancreas due to chronic pain syndrome or cancer). When used to treat a metabolic, enzymatic, or hormonal deficiency, the cells of the invention can be administered to numerous locations including, but not limited to, the site where the damaged or malfunctioning cells normally reside. For example, cells used to treat diabetes need not be delivered into the recipient's pancreas but can be provided beneath the kidney capsule or within an

implantable device. When spore-like cells or their progenitors are administered to the pancreas, where they have access to the growth factors and other compounds that normally influence islet cells, it is less important that they be administered in a differentiated state. However, when the cells are administered elsewhere (*e.g.*, beneath the kidney capsule or within an implantable device) it is more important that they be administered in a differentiated or committed state or administered together with factors that will promote their differentiation.

When spore-like cells are used in cell therapies, they can be administered just as more differentiated cells have been administered. For example, when spore-like cells are used to treat diabetes, they can be administered just as mature insulin-expressing cells have been administered (*e.g.*, by implantation under the renal capsule or within various implantable or extracorporeal devices). In fact, spore-like cells can be placed within a containment device and implanted, for example, within a patient's abdomen to treat a variety of disorders. This method of administration is particularly well suited for treating systemic disorders, such as those caused by an enzymatic imbalance. Implantation by way of containment devices is also useful when cells require protection from the patient's immune system.

Alternatively, as described below, spore-like cells and their progeny can be used in conjunction with tissue constructs (*i.e.*, any medium, structure or device that contains or supports (physically or chemically) biological material, including material that consists of or includes the novel cells of the present invention). A tissue construct can include a biocompatible solution (*e.g.* a saline solution) that includes cytokines, growth factors, and antibiotics, and materials or devices useful in reengineering damaged, diseased, or otherwise unhealthy tissue. The constructs can include support structures, such as a mesh, and/or a hydrogel. Together, the hydrogel and the spore-like cells of the invention form a hydrogel-spore-like cell composition. Similarly, a hydrogel combined with a progenitor cell forms a hydrogel-progenitor cell composition.

Spore-like cells can be combined with a liquid hydrogel that can be placed in a permeable, biocompatible support structure that is delivered to a patient (either before or after it is filled with the hydrogel-cell composition). As the hydrogel-cell composition fills the support structure, it assumes the structure's shape. When spore-

like cells proliferate and differentiate to such an extent that they form new tissue, the support structure guides the shape of the developing tissue. For example, the support structure can be formed in the shape of a bone (or a fragment thereof), a meniscus within a joint, an ear, an internal organ (or a portion thereof), or other tissue (*e.g.*, the skin). However, the support structure need not be strictly fashioned after naturally occurring tissue in every case. For example, the support structure can be shaped in a way that simply facilitates delivery of spore-like cells to a patient. For example, the support structure can be shaped to fit under the renal capsule or within some other organ or cavity (*e.g.*, the support structure can be shaped to lie within a portion of the gastrointestinal tract or to fill a space once occupied by tissue, such as the spaces created when a tumor is surgically removed or when a tissue has been destroyed following trauma, ischemia, or an autoimmune response).

In some instances, including instances where spore-like cells are administered in the course of cell or gene therapy, spore-like cells can be administered without containment devices, hydrogels, or support structures. It is well within the ability of one of ordinary skill in the art to determine when spore-like cells should be confined within a space dictated by a support structure and when they should not. For example, one of ordinary skill in the art would recognize that when treating respiratory distress syndrome (RDS) with spore-like cells that are made to secrete surfactant, or that differentiate into cells that secrete surfactant, the surfactant, which reduces surface tension within the alveoli, must be supplied locally. Thus, spore-like cells obtained, *e.g.*, from healthy human lung tissue, for example, taken from a healthy portion of a lung of the patient or from a donor, are delivered to the diseased portions of the patient's lungs, *e.g.*, by inhalation in the form of an aerosol.

Cells obtained from an immunologically distinct donor can illicit an immune response, in which case, the patient (the recipient of the cells) can be treated with standard immunosuppressant therapy (*e.g.*, with cyclosporine and/or steroid hormones). While immunosuppression is commonly required when transplanting typical mature cells (*e.g.*, when transplanting an organ such as the liver or kidney), it should not be required when administering the unique cells of the invention. These cells, particularly spore-like cells, are so undifferentiated that they may not express surface antigens and thus would not elicit an immune response, even if isolated from a

different species or a different individual of the same species as the patient who will receive the cells.

C. Administration of Cells via Hydrogel

The novel cell types described herein can be administered to a patient by way of a composition that includes a hydrogel. For example, a hydrogel can be combined with spore-like cells, their non-terminally differentiated progeny, one or more types of differentiated cells, or a combination of these cell types. This cell-hydrogel mixture can be applied directly to a tissue that has been damaged or to a device that is then implanted in, or attached to, a patient. For example, as described in U.S.S.N. 08/747,036, a hydrogel-cell mixture can simply be applied to a desired surface of a tissue (by, *e.g.*, brushing, dripping, or spraying the mixture onto the surface) or poured into or otherwise made to fill a desired cavity or device. The hydrogel provides a thin matrix or scaffold within which the cells (*e.g.*, the spore-like cells) adhere and function (by, *e.g.*, differentiating, proliferating, or secreting a biologically active substance such as insulin). The methods of administration described here are especially well suited when the tissue associated with a patient's disorder has an irregular shape or when the cells are applied at a distant site (*e.g.*, when spore-like cells that are made to express insulin or that differentiate into cells that express insulin are placed beneath the renal capsule, elsewhere in the abdomen, or within an extracorporeal device to treat diabetes).

Alternatively, the hydrogel-cell mixture can be introduced into a permeable, biocompatible support structure so that the mixture essentially fills all, or a desired portion of, the support structure and, as it solidifies, assumes the support structure's shape. Thus, the support structure can guide the development and shape of tissues that mature from cells (*e.g.*, spore-like cells and their progeny) within it. As described further below, the support structure can be provided to a patient either before or after being filled with the hydrogel-cell mixture. For example, the support structure can be placed within a tissue (*e.g.*, a damaged area of the skin, liver, lung, or the skeletal system) and subsequently filled with the hydrogel-cell composition using a syringe, catheter, or other suitable device. When desirable, the shape of the support structure can be made to conform to the shape of the damaged tissue. In the following

subsections, suitable support structures, hydrogels, and delivery methods are described (cells suitable for use are described above).

1. Hydrogels

The hydrogels used to practice this invention should be biocompatible, capable of sustaining living cells (*i.e.*, nontoxic to the cells combined with it), and, preferably, should solidify rapidly *in vivo* (*e.g.*, in about five minutes after being delivered to the support structure). Large numbers of cells (*e.g.*, spore-like cells) can be distributed evenly within a hydrogel; a hydrogel can support approximately 5×10^6 cells/ml. The hydrogel should contain, but in most cases not isolate, the cells within it. Nutrients should be able to diffuse through the hydrogel to reach the cells and waste products or other secreted substances should be able to reach the patient.

A variety of different hydrogels can be used to practice the invention. These include, but are not limited to: (1) temperature dependent hydrogels that solidify or set around body temperature (*e.g.*, PLURONICS™); (2) hydrogels cross-linked by ions (*e.g.*, sodium alginate); (3) hydrogels set by exposure to either visible or ultraviolet light (*e.g.*, polyethylene glycol polylactic acid copolymers with acrylate end groups); and (4) hydrogels that are set or solidified upon a change in pH (*e.g.*, TETRONICS™).

Materials that can be used to form these different hydrogels include, but are not limited to, polysaccharides such as alginate, polyphosphazenes, and polyacrylates, which are cross-linked ionically, block copolymers such as PLURONICS™ (also known as POLOXAMERS™), which are poly(oxyethylene)-poly(oxypropylene) block polymers solidified by changes in temperature, TETRONICS™ (also known as POLOXAMINES™), which are poly(oxyethylene)-poly(oxypropylene) block polymers of ethylene diamine solidified by changes in pH.

a. Ionic Hydrogels

Ionic polysaccharides, such as alginates or chitosan, can be used to suspend living cells, including spore-like cells and their progeny. These hydrogels can be produced by cross-linking the anionic salt of alginic acid, a carbohydrate polymer isolated from seaweed, with ions, such as calcium cations. The strength of the hydrogel increases with either increasing concentrations of calcium ions or alginate. U.S. Patent No. 4,352,883 describes the ionic cross-linking of alginate with divalent cations, in water, at room temperature, to form a hydrogel matrix.

Spore-like cells, their progeny, or a mixture of cells including either of these cell types, are mixed with an alginate solution, the solution is delivered to an already implanted support structure, which then solidifies in a short time due to the presence of physiological concentrations of calcium ions *in vivo*. Alternatively, the solution is delivered to the support structure prior to implantation and solidified in an external solution containing calcium ions.

In general, the polymers described here are at least partially soluble in aqueous solutions (*e.g.*, water, aqueous alcohol solutions that have charged side groups, or monovalent ionic salts thereof). There are many examples of polymers with acidic side groups that can be reacted with cations (*e.g.*, poly(phosphazenes), poly(acrylic acids), and poly(methacrylic acids)). Examples of acidic groups include carboxylic acid groups, sulfonic acid groups, and halogenated (preferably fluorinated) alcohol groups. Examples of polymers with basic side groups that can react with anions are poly(vinyl amines), poly(vinyl pyridine), and poly(vinyl imidazole).

Polyphosphazenes are polymers with backbones consisting of nitrogen and phosphorous atoms separated by alternating single and double bonds. Each phosphorous atom is covalently bonded to two side chains. Polyphosphazenes that can be used have a majority of side chains that are acidic and capable of forming salt bridges with di- or trivalent cations. Examples of acidic side chains are carboxylic acid groups and sulfonic acid groups.

Bioerodible polyphosphazenes have at least two different types of side chains: acidic side chains that can form salt bridges with multivalent cations and side chains that hydrolyze *in vivo* (*e.g.*, imidazole groups, amino acid esters, glycerol, and glucosyl). Bioerodible or biodegradable polymers (*i.e.*, polymers that dissolve or

degrade within a period that is acceptable in the desired application (usually *in vivo* therapy)), will degrade in less than about five years and most preferably in less than about one year, once exposed to a physiological solution of pH 6-8 having a temperature of between about 25°C and 38° C. Hydrolysis of the side chain results in erosion of the polymer. Examples of hydrolyzing side chains are unsubstituted and substituted imidizoles and amino acid esters in which the side chain is bonded to the phosphorous atom through an amino linkage.

Methods for synthesis and the analysis of various types of polyphosphazenes are described in U.S. Patent Nos. 4,440,921, 4,495,174, and 4,880,622. Methods for synthesizing other polymers described above are known to those of ordinary skill in the art. *See*, for example Concise Encyclopedia of Polymer Science and Engineering, J.I. Kroschwitz, Ed., John Wiley and Sons, New York, NY, 1990. Many polymers, such as poly(acrylic acid), alginates, and PLURONICS™ are commercially available.

Water soluble polymers with charged side groups are cross-linked by reacting the polymer with an aqueous solution containing multivalent ions of the opposite charge, either multivalent cations if the polymer has acidic side groups, or multivalent anions if the polymer has basic side groups. Cations for cross-linking the polymers with acidic side groups to form a hydrogel include divalent and trivalent cations such as copper, calcium, aluminum, magnesium, and strontium. Aqueous solutions of the salts of these cations are added to the polymers to form soft, highly swollen hydrogels.

Anions for cross-linking the polymers to form a hydrogel include divalent and trivalent anions such as low molecular weight dicarboxylate ions, terepthalate ions, sulfate ions, and carbonate ions. Aqueous solutions of the salts of these anions are added to the polymers to form soft, highly swollen hydrogels, as described with respect to cations.

Those of ordinary skill in the art are well able to adjust the size of the polymers formed to accommodate various uses and needs. For example, when the polymer in the hydrogel ranges from 10 to 18.5 kDa, most antibodies will not pass into the hydrogel, but nutrients will. When desired, smaller polymers can be made that give rise to hydrogels of higher density (*i.e.*, smaller pores).

b. Temperature-Dependent Hydrogels

Temperature-dependent, or thermosensitive, hydrogels can also be used in the methods of the invention. These hydrogels have so-called "reverse gelation" properties (*i.e.*, they are liquids at or below room temperature and gel when warmed to higher temperatures (*e.g.*, body temperature)). Thus, these hydrogels can be easily applied at or below room temperature as a liquid and automatically form a semi-solid gel when warmed to body temperature. As a result, these gels are especially useful when the support structure is first implanted into a patient, and then filled with a hydrogel-cell composition. Examples of such temperature-dependent hydrogels are PLURONICS™ (BASF-Wyandotte), such as polyoxyethylene-polyoxypropylene F-108, F-68, and F-127, poly (N-isopropylacrylamide), and N-isopropylacrylamide copolymers.

These copolymers can be manipulated by standard techniques to affect their physical properties such as porosity, rate of degradation, transition temperature, and degree of rigidity. For example, the addition of low molecular weight saccharides in the presence and absence of salts affects the lower critical solution temperature (LCST) of typical thermosensitive polymers. In addition, when these gels are prepared at concentrations ranging between 5% and 25% (W/V) by dispersion at 4°C, the viscosity and the gel-sol transition temperature are affected, the gel-sol transition temperature being inversely related to the concentration. These gels have diffusion characteristics capable of allowing spore-like cells and their progeny to survive and be nourished.

U.S. Patent No. 4,188,373 describes using PLURONIC™ polyols in aqueous compositions to provide thermal gelling aqueous systems. U.S. Patent Nos. 4,474,751, '752, '753, and 4,478,822 describe drug delivery systems that utilize thermosetting polyoxyalkylene gels. With these systems, any of which can be used to deliver the novel cells of the present invention to a patient, both the gel transition temperature and the rigidity of the gel can be modified by adjusting the pH or the ionic strength, as well as by the concentration of the polymer.

c. pH-Dependent Hydrogels

Other hydrogels suitable for use in the methods of the invention are pH-dependent. These hydrogels are liquids at, below, or above specific pH values, and gel when exposed to specific pHs, for example, 7.35 to 7.45, the normal pH range of extracellular fluids within the human body. Thus, these hydrogels can be easily delivered to an implanted support structure as a liquid and automatically form a semi-solid gel when exposed to physiological pH. Examples of such pH-dependent hydrogels are TETRONICS™ (BASF-Wyandotte) polyoxyethylene-polyoxypropylene polymers of ethylene diamine, poly(diethyl aminoethyl methacrylate-g-ethylene glycol), and poly(2-hydroxymethyl methacrylate). These copolymers can be manipulated by standard techniques to affect their physical properties.

d. Light Solidified Hydrogels

Other hydrogels that can be used to administer spore-like cells or their progeny are solidified by either visible or ultraviolet light. These hydrogels are made of macromers including a water-soluble region, a biodegradable region, and at least two polymerizable regions (*see, e.g.*, U.S. Patent No. 5,410,016). For example, the hydrogel can begin with a biodegradable, polymerizable macromer including a core, an extension on each end of the core, and an end cap on each extension. The core is a hydrophilic polymer, the extensions are biodegradable polymers, and the end caps are oligomers capable of cross-linking the macromers upon exposure to visible or ultraviolet light, for example, long wavelength ultraviolet light.

Examples of such light solidified hydrogels include polyethylene oxide block copolymers, polyethylene glycol polylactic acid copolymers with acrylate end groups, and 10K polyethylene glycol-glycolide copolymer capped by an acrylate at both ends. As with the PLURONIC™ hydrogels, the copolymers comprising these hydrogels can be manipulated by standard techniques to modify their physical properties (such as rate of degradation, differences in crystallinity, and degree of rigidity).

2. Preparation of Hydrogel-Cell Mixtures

Once a hydrogel of choice (*e.g.*, a thermosensitive polymer at between 5% and 25% (W/V), or an ionic hydrogel such as alginate dissolved in an aqueous solution (*e.g.*, a 0.1 M potassium phosphate solution, at physiological pH, to a concentration between 0.5% to 2% by weight) is prepared, isolated spore-like cells or their progeny (with or without differentiated cells) are suspended in the polymer solution. If desired, the concentration of the cells can mimic that of the tissue to be generated. For example, the concentration of cells can range between 10 and 100 million cells/ml (*e.g.*, between 20 and 50 million cells/ml or between 50 and 80 million cells/ml). Of course, the optimal concentration of cells to be delivered into the support structure can be determined on a case by case basis, and will vary depending on cell type and the region of the patient's body into which the support structure is implanted or onto which it is applied. To optimize the procedure (*i.e.*, to provide optimal viscosity and cell number), one need only vary the concentrations of the cells or the hydrogel.

3. Support Structures

The support structure is a permeable structure having pore-like cavities or interstices that shape and support the hydrogel-cell mixture. For example, the support structure can be a porous polymer mesh, or a natural or synthetic sponge. The porosity of the support structure should be such that nutrients can diffuse into the structure, thereby effectively reaching the cells inside, and products produced by the cells (either products that can harm the cells, such as waste products, or products that can aid the patient (*e.g.*, insulin, somatostatin, alpha-galactosidase A)) can diffuse out of the structure.

The support structure can be shaped to conform to the space in which new tissue is desired. For example, the support structure can be shaped to conform to the shape of an area of the skin that has been burned or the portion of cartilage or bone that has been lost. Depending on the material from which it is made, the support structure can be shaped by cutting, molding, casting, or any other method that produces a desired shape (as described below, in some instances, the support structure can be shaped by hand). Moreover, the shaping process can occur either before or after the support structure is filled with the hydrogel-cell mixture. For example, a

support structure can be filled with a hydrogel-cell mixture and, as the hydrogel hardens, molded into a desired shape by hand.

As the hydrogel solidifies, it will adopt the flexibility and resiliency of the support structure, which can be important for accommodation of compressive and tensile forces. Thus, for example, replaced skin will accommodate tensile forces associated with pulling and stretching, as well as compressive forces associated with weight bearing, as occurs, for example, on the soles of the feet. The flexibility and resiliency of the support structure also provides greater ease of administration than do many currently available skin replacement methods in which the tissue is extremely delicate and must therefore be handled with the utmost care.

Like the hydrogel itself, the support structure is biocompatible (*i.e.*, it is not toxic to the spore-like cells suspended therein) and can be biodegradable. Thus, the support structure can be formed from a synthetic polymer such as a polyanhydride, polyorthoester, or polyglycolic acid. The polymer should provide the support structure with an adequate shape and promote cell survival and, when required proliferation and differentiation, by allowing nutrients to reach the cells. Additional factors, such as growth factors, other factors that induce differentiation or dedifferentiation, secretion products, immunomodulators, anti-inflammatory agents, regression factors, biologically active compounds that promote innervation or enhance the lymphatic network, and drugs, can be incorporated into the polymer support structure.

An example of a suitable polymer is polyglactin, which is a 90:10 copolymer of glycolide and lactide, and is manufactured as VICRYL™ braided absorbable suture (Ethicon Co., Somerville, NJ). Polymer fibers (such as VICRYL™) can be woven or compressed into a felt-like polymer sheet, which can then be cut into any desired shape. Alternatively, the polymer fibers can be compressed together in a mold that casts them into the shape desired for the support structure. In some cases, additional polymer can be added to the polymer fibers as they are molded to revise or impart additional structure to the fiber mesh. For example, a polylactic acid solution can be added to this sheet of polyglycolic fiber mesh, and the combination can be molded together to form a porous support structure. The polylactic acid binds the crosslinks of the polyglycolic acid fibers, thereby

coating these individual fibers and fixing the shape of the molded fibers. The polylactic acid also fills in the spaces between the fibers. Thus, porosity can be varied according to the amount of polylactic acid introduced into the support. The pressure required to mold the fiber mesh into a desirable shape can be quite moderate. All that is required is that the fibers are held in place long enough for the binding and coating action of polylactic acid to take effect.

Alternatively, or in addition, the support structure can include other types of polymer fibers or polymer structures produced by techniques known in the art. For example, thin polymer films can be obtained by evaporating solvent from a polymer solution. These films can be cast into a desired shape if the polymer solution is evaporated from a mold having the relief pattern of the desired shape. Polymer gels can also be molded into thin, permeable polymer structures using compression molding techniques known in the art.

Many other types of support structures are also possible. For example, the support structure can be formed from sponges, foams, corals, or biocompatible inorganic structures having internal pores, or mesh sheets of interwoven polymer fibers. These support structures can be prepared using known methods.

4. Application of the Support Structure

Any of the liquid hydrogel-cell mixtures described above can be placed in any of the permeable support structures (also described above). Fig. 13 is a schematic of a filled support structure in cross-section. This structure, as illustrated, is suitable for application of compositions containing spore-like cells, or their progeny, to the skin. The support structure **10** is formed from a bilayered mesh of interwoven polymer fibers **12** having epidermal layer **12a** and dermal layer **12b**. The spaces between the fibers form interconnected pores **14** that are filled with liquid hydrogel-cell mixture. Within a short time of placing the mixture in the support structure (*e.g.*, in approximately three to five minutes), hydrogel **16** solidifies, thereby keeping suspended cells **18** within pores **14** of support structure **10**. The solidified hydrogel **16** helps maintain the viability of the cells by allowing diffusion of nutrients (including growth and differentiation factors) and waste products through the interconnected pores of the support structure. The ultimate result is the growth of new skin and its engraftment to the patient's body.

The liquid hydrogel-cell mixture can be delivered to the shaped support structure either before or after the support structure is implanted in or applied to a patient. The specific method of delivery will depend on whether the support structure is sufficiently "sponge-like" for the given viscosity of the hydrogel-cell composition, *i.e.*, whether the support structure easily retains the liquid hydrogel-cell mixture before it solidifies. Sponge-like support structures can be immersed within, and saturated with, the liquid hydrogel-cell mixture, and subsequently removed from the mixture. The hydrogel is then allowed to solidify within the support structure. The hydrogel-cell-containing support structure is then implanted in or otherwise administered to the patient.

The support structure can also be applied to the patient before the hydrogel completely solidifies. Alternatively, a sponge-like support structure can be injected with the liquid hydrogel-cell mixture, either before or after the support structure is implanted in or otherwise administered to the patient. The hydrogel-cell mixture is then allowed to solidify.

The volume of the liquid hydrogel-cell mixture injected into the support structure is typically less than, but somewhat comparable to, the volume of the support structure, *i.e.*, the volume of the desired tissue to be grown.

Support structures that do not easily retain the liquid composition require somewhat different methods. In those cases, for example, the support structure is immersed within and saturated with the liquid hydrogel-cell mixture, which is then allowed to partially solidify. Once the cell-containing hydrogel has solidified to the point where the support structure can retain the hydrogel, the support structure is removed from the partially solidified hydrogel, and, if necessary, partially solidified hydrogel that remains attached to the outside of the support structure is removed (*e.g.*, scraped off the structure).

Alternatively, the liquid hydrogel-cell mixture can be delivered into a mold containing the support structure. For example, the liquid hydrogel-cell mixture can be injected into an otherwise fluid-tight mold that contains the support structure and matches its outer shape and size. The hydrogel is then solidified within the mold, for example, by heating, cooling, light-exposure, or pH adjustment, after which, the

hydrogel-cell-containing support structure can be removed from the mold in a form that is ready for administration to a patient.

In other embodiments, the support structure is implanted in or otherwise administered to the patient (*e.g.*, placed over the site of a burn or other wound, placed beneath the renal capsule, or within a region of the body damaged by ischemia), and the liquid hydrogel-cell mixture is then delivered to the support structure. As noted above, the hydrogel-cell mixture can be delivered to the support using any simple device, such as a syringe or catheter, or merely by pouring, dripping, or brushing a liquid gel onto a support structure (*e.g.*, a sheet-like structure).

Here again, the volume of hydrogel-cell composition added to the support structure should approximate the size of the support structure (*i.e.*, the volume displaced by the desired tissue to be grown). The support structure provides space and a structural template for the injected liquid hydrogel-cell mixture. In the event some of the hydrogel-cell mixture leaks from the support structure prior to solidifying, existing tissue beneath or around the support structure will constrain the liquid hydrogel-cell mixture until it gels.

In any of the above cases, the hydrogel is solidified using a method that corresponds to the particular hydrogel used (*e.g.*, gently heating a composition including a PLURONIC™ temperature-sensitive hydrogel).

To apply or implant the support structure, the implantation site within the patient can be prepared (*e.g.*, where the support structure is applied to the skin, the area can be prepared by debridement), and the support structure can be implanted or otherwise applied directly at that site. If necessary, during implantation, the site can be cleared of bodily fluids such as blood (*e.g.*, with a burst of air or suction).

5. Administration of Cells with Specific Skin Replacement Therapies

Spore-like cells, their non-terminally differentiated progeny, one or more types of differentiated cells, or a combination of these cell types (*e.g.*, spore-like cells and skin progenitor cells or spore-like cells and keratinocytes or fibroblasts) can also be administered by way of existing skin replacement therapies. Including the novel cells of the present invention improves existing methods because the present cells can differentiate into cell types and structures that would not otherwise be present, and thereby improve the structure, functions, and appearance of the replacement skin.

a. VICRYL™ Mesh

Spore-like cells and skin progenitor cells can be administered in connection with dermal tissue replacements that are placed beneath meshed, split-thickness skin grafts. To construct the tissue replacement, human dermal fibroblasts are isolated from neonatal foreskin as follows. Epidermis and dermis are separated by incubation in 0.25% trypsin with 0.2% ethylenediaminetetraacetic acid for 1-2 hours at 37°C. The dermis is minced and digested with collagenase B, and the tissue digest is filtered through sterile gauze to remove debris. Fibroblasts are maintained in Dulbecco's modified eagle's medium (DMEM) and passaged when cells reach 80-90% confluence. Cells are removed from flasks and resuspended for seeding at a concentration of 4×10^6 cells/ml.

Spore-like cells or skin progenitor cells are isolated and cultured as described herein, and resuspended for seeding at a concentration of approximately 4×10^6 cells/ml.

Dermal grafts are then prepared by seeding viable fibroblasts and spore-like cells and/or skin progenitor cells in a minimum volume of DMEM onto each 4 cm^2 area of VICRYL™ mesh (Ethicon Inc., Somerville, NJ). The cells readily attach *in vitro* to the mesh fibers, and they become confluent in 2 to 3 weeks (*i.e.*, all mesh openings are covered by cells and tissue matrix, according to assay by inverted phase microscopy).

The patient's wound can be excised to subcutaneous fat, to fascia, or to deep dermis and fat. Hemostatis can be achieved with topical thrombin-epinephrine solution and electrocoagulation. The graft, prepared as described above, is placed on the prepared site and can be affixed to the wound margins with staples or sutures. If desired, a hydrogel can be used together with the Vicryl™ mesh.

b. Collagen-based Skin Substitutes

Spore-like cells, skin progenitor cells, or compositions of cells containing them can also be used in conjunction with numerous permanent dermal skin replacements that include, for example, resorbable synthetic composites containing collagen and chondroitin-6-sulfate (*e.g.*, glycosaminoglycan (GAG) dermal membranes are described by Burke *et al.*, *Ann. Surg.* 194:413-428, 1981; Yannas *et*

al., *Science* 214:174-176, 1982; and Yannas *et al.*, U.S. Patent No. 4,060,081). The present cells, alone or in combination with one another or with one or more differentiated cell types can also be used in lieu of keratinocytes in the dermal collagen-chondroitin-6-sulfate membrane described in EP 0 363 400 B1 or in the collagen matrices that include transforming growth factor-α (TGF-α) described in U.S. Patent No. 5,800,811. TGF-α impregnated collagen matrices may be advantageous in that they are thought to inhibit inflammatory processes while promoting angiogenesis and histogenesis.

c. Multilayer Skin Substitutes

Spore-like cells, skin progenitor cells, or compositions of cells containing them are especially well suited for use with multilayer skin substitutes. Spore-like cells or progenitor cells isolated from the dermis can be incorporated in the lower or "dermal" layer, where they would subsequently differentiate into adnexal structures, and spore-like cells or progenitor cells isolated from the epidermis can be incorporated in the upper or "epidermal" layer, where they would subsequently differentiate into melanocytes cells, keratinocytes, and Merkel cells. Several multilayer skin equivalents that can be used in conjunction with compositions that include the cells of the present invention are described in WO 97/41208.

d. *In Vivo* Testing

Numerous *in vivo* models of wound healing are available to evaluate and optimize the performance of spore-like cells, skin progenitor cells, and compositions containing them. For example, the ability to repair a burn can be tested in domestic outbred swine. The swine are anaesthesized by ketamine hydrochloride, followed by inhalation of a mixture of halothane, nitrous oxide, and oxygen. A portion of their skin is shaved, washed twice with BETADINE™, and washed once with 70% alcohol. Wounds that are 1 mm deep are then created with a Brown dermatome on the lateral side. Wounds of this depth correspond to deep second degree burns and remove all of the epidermis and most of the dermis. Of course, the depth of the injury can be increased to mimic a more severe burn or decreased to mimic a less severe burn. The size of the wounds can also be varied. Typically, a wound is approximately 2.5

inches square. Following surgery, the pigs are treated with analgesics to alleviate their pain.

A second reliable skin injury model has been developed in pigs to study the use of hyaluronidase in the treatment of intravenous extravasation injuries (Raszka *et al., J. Perinatol.* 10:146-149, 1990). In this model, the flanks of recently weaned Yorkshire pigs are shaved and then cleaned with alcohol and povidone-iodine solutions. A subcutaneous injection of $CaCl_2$ (300 mEq/L; 2.0 ml) is then given, which causes ulceration (an area of full thickness skin necrosis greater than 0.5 cm^2).

Murine wound healing models are also available. For example, Moore *et al.* (*Br. J. Cancer* 66:1037-1043, 1992) describe a model that can be used to compare patterns of damage to skin and its supporting vasculature following treatment by hyperthermia and photodynamic therapy. More straight-forward injury models are also available. For example, rodents used in studies of hyperthermia have received burns by exposure to a heat source. For example, an area of the skin can be injured by exposure to hot water (98°C) for a given number of seconds (9 seconds; as in Farriol *et al., Burns* 20:496-498, 1994). As in the models above, the animals are medicated to alleviate their pain.

EXAMPLES

The present invention will be further understood by reference to the following non-limiting examples.

Example 1

Spore-like cells were isolated from human blood as follows. Samples of whole blood (approximately five ml each) were acquired from adult humans and placed in a tube containing an anti-coagulant. The samples were then centrifuged at 1200 rpm for approximately five minutes. The supernatant was removed and the resulting pellet was resuspended in DMEM/F-12 medium (15 ml) supplemented with a combination of the following hormones and nutrients: glucose (23 mM), transferrin (10 mg/ml), insulin (20 mg/ml), putricine (10 mM), selenium (100 nM), progesterone (10 nM) (Life Technologies, Baltimore, MD), EGF (20 ng/ml), and bFGF (20 ng/ml) (Collaborative Biomedical Products, Chicago, IL). The resulting suspension was

transferred to 75 cm^2 tissue culture flasks and incubated in 5% CO_2 at 37°C. The media were changed every 3-4 days. Cells were passaged every 7-9 days. Initially, these culture flasks appeared to contain many hematopoeitic cells (*e.g.*, red blood cells), but over time (usually, a matter of several days), these cells disappeared, leaving only spore-like cells.

After several days in culture, the spore-like cells multiplied and coalesced to form clusters of cells. Trypan blue exclusion revealed cell viability to be greater than 90%. Figs. 11 and 12A are photographs of cultures that include undifferentiated spore-like cells isolated from adult human blood. The cells shown in Fig. 12A were isolated seven days earlier and are viewed with phase contrast microscopy. Immunofluorescent staining was then performed. At this time, some of the cells expressed nestin (see Fig. 12B).

Example 2

Spore-like cells were isolated from the skin of an adult rodent as follows. Excisional biopsies of the skin of adult Fisher rats were made under sterile conditions. The biopsied tissue, which included the dermis and epidermis, was placed in a petri dish containing cold (50°C) phosphate buffered saline (PBS) and antibiotics (penicillin (50 mU/ml) and streptomycin (90 mg/ml)). The epidermis was scraped with a #11 scalpel to disassociate epidermal cells, and the tissue was then transferred to a second petri dish (also containing cold PBS and antibiotics) where the dermis was scraped with a #11 scalpel. The cells that were dissociated were then centrifuged at 1200 rpm (GLC-2B, Sorvall, Wilmington, DE) for five minutes and resuspended in 10 ml of 0.05% trypsin (Life Technologies, Baltimore, MD). Following resuspension in trypsin, the tissue was incubated at 37°C for five minutes. Ten ml of Dulbecco's Modified Eagle Medium (DMEM)/F-12 containing 10% heat inactivated fetal bovine serum (FBS) (Life Technologies, Baltimore, MD) was added to deactivate the trypsin.

The tissue was then triturated, first with a normal bore Pasteur pipette and subsequently with a series of fire polished pipettes having bores reduced to about 15 μm. The number of pipettes required can vary, depending upon a number of factors, including the initial size of the tissue fragments obtained by scraping the excised skin and how frequently the pipettes become clogged with tissue. Trituration

was carried out until the tissue was dispersed as a fine suspension. The suspension was then centrifuged at 1200 rpm (GLC-2B, Sorvall, Wilmington, DE) for five minutes. The supernatant was removed and the pellet was resuspended in 15 ml of DMEM/F-12 medium supplemented with a hormone mixture containing glucose (23 mM), transferrin (10 mg/ml), insulin (20 mg/ml), putricine (10 mM), selenium (100 nM), progesterone (10 nM) (Life Technologies, Baltimore, MD), EGF (20 ng/ml) and bFGF (20 ng/ml) (Collaborative Biomedical Products, Chicago, IL). The suspension was transferred to 75 cm^2 tissue culture flasks (Collaborative Biomedical Products, Chicago, IL) and incubated at 37°C in 5% CO_2. The media was changed every three days and cells were passaged every 7-9 days. The cells that attached to the tissue culture flask appeared to differentiate more readily.

Spore-like cells isolated from the skin will differentiate upon exposure to the processes and basal nutrient media described in U.S. Patent No. 5,292,655. Alternatively, growth factors that cause spore-like cells to mitose (*e.g.*, epidermal growth factor (EGF), basic fibroblast growth factor (bFGF) and other cytokines) can be applied to help maintain the cells in an undifferentiated state. For example, the isolated cells can be cultured in Dulbecco's Modified Eagle's Medium (DMEM) supplemented with a hormone mixture containing glucose, transferrin, insulin, putricine, selenium, progesterone, EGF, and bFGF.

Spore-like cells were also isolated from excisional biopsies of the skin of adult pigs according to the same protocol described here for the adult rat.

Spore-like cells and skin progenitor cells isolated and cultured as described herein can differentiate in culture. The progenitor cells have been seen to form an unidentified adnexal skin structure, a primitive adnexal gland. Without limiting the invention to cells that differentiate by a particular mechanism, it is believed that progenitor cells may develop along various and committed lineages depending on the cues they receive from neighboring cells; it may be that a concerted and highly interactive process directs the progenitor cell down a particular differentiation pathway.

A biocompatible, biodegradable, reverse-thermosensitive copolymer gel can be obtained by preparing a 30% weight/volume solution of a PLURONIC™ F127, F68 block copolymer (available from BASF). The solution remains in a liquid state at less than 15°C, and solidifies within 5 to 10 minutes as the temperature is increased to over 15°C. Skin progenitor cells isolated as described above are added to the hydrogel mixture to generate a final cellular density of about 2×10^6 to 6×10^7 cells per ml. Such a mixture can be delivered into a permeable support structure.

Example 3

Spore-like cells were isolated from adult rat heart according to the protocol described in Example 2. The newly isolated cells, which are shown in Fig. 3A, include undifferentiated spore-like cells. After culturing the spore-like cells for three days, early myocardial cells can be seen (Fig. 3B), and after two weeks in culture, Purkinje-like structures can be seen (Fig. 3C).

Example 4

Spore-like cells were isolated from adult rat intestine according to the protocol described in Example 2. The newly isolated cells, as shown in Fig. 4A, include undifferentiated spore-like cells. After culturing the cells for three days, clusters of small intestinal cells (Fig. 4B) and autonomic neurons (Fig. 4C) can be seen.

Example 5

Spore-like cells were isolated from an adult rat bladder according to the protocol described in Example 2. The newly isolated cells, which are shown in Fig. 5A, include undifferentiated spore-like cells. After culturing the spore-like cells for two days, the isolated spore-like cells, or their progeny, appear to be differentiating into mature bladder cells (Fig. 5B).

Example 6

Spore-like cells were isolated from an adult rat kidney according to the protocol described in Example 2. Cells newly isolated from the kidney of an adult rat, which are shown in Fig. 6A, include undifferentiated spore-like cells. After culturing the spore-like cells for three days, aggregates of cells resembling kidney structures can be seen (Fig. 6B).

Example 7

Spore-like cells were isolated from an adult rat liver according to the protocol described in Example 2. Because the liver is highly vascularized, the intact tissue was washed with PBS. Cells newly isolated from the liver of an adult rat, which are shown in Figs. 7A and 7C, include undifferentiated spore-like cells. After culturing the spore-like cells for three days, an aggregate of cells resembling a differentiating liver structure can be seen (Fig. 7B). After culturing the spore-like cells for seven days, cells resembling hepatocytes can be seen (Fig. 7D).

Example 8

Spore-like cells were isolated from adult mammalian lungs according to the protocol described in Example 2. Spore-like cells were isolated from the lungs of adult rats (see Figs. 8A-8C) and sheep (see Fig. 8D). The newly isolated cells shown in Fig. 8A include undifferentiated spore-like cells. After six weeks in culture, alveolar-like cells can be seen (Figs. 8B and 8C). After culturing the spore-like cells for 30 days, spore-like cells isolated from an adult sheep have formed alveolar-like structures (Fig. 8D) similar to those seen in the lungs of adult cats (Fig. 8E; Histology, F. Hammersen, Ed., Urban & Schwarzenberg, Baltimore-Munich, 1980, Fig. 321).

Example 9

Spore-like cells were isolated from adult rat adrenal glands according to the protocol described in Example 2. Undifferentiated spore-like cells isolated from the adrenal gland of an adult rat can be seen at Day 0 in Figs. 9A and 9B (see the arrows). After culturing the spore-like cells for two days, primitive adrenal cells can be seen (Figs. 9C and 9D).

Example 10

Spore-like cells were isolated from the pancreas of an adult human and from the pancreas of an adult rat. The dissections were carried out in 10% cold fetal serum albumin according to the protocol described in Example 2. Significantly, spore-like cells have been isolated from a portion of the rat pancreas that remained after the islets were removed by ductal injection of collagenase (as described, for example, by Sutton *et al.*, *Transplantation*, 42:689-691, 1986).

Islet-like structures that formed in cultures of spore-like cells isolated from islet-free pancreatic tissue are shown in Figs. 10A-10C. After six days in culture, more than 100 islet-like structures were present per field (see Figs. 10A and 10B), even though the spore-like cells first placed in culture were isolated from a tissue from which the islets had been removed. When the islet-like structures that nevertheless developed were immunostained, insulin expression can be seen (Fig. 10C).

Example 11

To isolate spore-like cells and retinal progenitor cells from the retina of an adult fisher rat, the animal's eyes were removed under sterile conditions and cut in half using a #11 scalpel. The retinal tissue was then scraped and disassociated with a # 11 scalpel in a petri dish containing cold (50°C) phosphate buffered saline with antibiotics (penicillin (50 mU/ml) and streptomycin (90 mg/ml)). The dissociated tissue was then centrifuged at 1200 rpm (GLC-2B, Sorvall, Wilmington, DE) for five minutes and resuspended in 10 ml of 0.05% trypsin (Life Technologies, Baltimore, MD) for an additional five minutes at 37°C. Ten ml of Dulbecco's Modified Eagle Medium (DMEM)/F-12, containing 10% heat inactivated fetal bovine serum (FBS) (Life Technologies, Baltimore, MD) was then added to deactivate the trypsin. The samples were triturated with a normal bore pasteur pipette, followed by sequential trituration with fire polished, reduced bore pasteur pipettes, the smallest being about 15 microns. Trituration was carried out until the tissue was dispersed as a fine suspension, and the solution was then centrifuged at 1200 rpm (GLC-2B, Sorvall, Willmington, DE) for five minutes. The supernatant was removed, and the resulting

pellet was resuspended in 15 ml of DMEM/F-12 medium (Life Technologies, Baltimore, MD) supplemented with the hormone mixture described in Example 2. The suspension was transferred to 75 cm^2 tissue culture flasks (Collaborative Biomedical Products, Chicago, IL) and incubated at 37°C in 5% CO_2. The media was changed every three days, and cells were passaged every 7-9 days.

The isolation procedure described above can also be carried out following enucleation of the eye from a deceased human donor. This procedure is well established and is carried out under sterile conditions. To remove retinal tissue from an intact eye, vitreous humor is removed (vitrectomy) and cells, generally from the periphery of the retina, are removed using standard retinal biopsy techniques.

Spore-like cells and retinal progenitors isolated and cultured as described herein can differentiate in culture. These cells have been seen to assume morphologies reminiscent of both rods and cones. As with spore-like cells isolated from other sources, spore-like cells isolated from the retina and their progeny may develop along various and committed lineages depending on the cues they receive from neighboring cells; a concerted and highly interactive process directs the progenitor cell down a particular differentiation pathway.

Retinal repair can be assessed *in vivo* using techniques routinely practiced by those of ordinary skill in the art in various animal models (*e.g.*, rabbit). For example, following implantation of spore-like cells, retinal progenitor cells, or compositions containing both, one can record cortical evoked responses to light using standard electrophysiology techniques. Recordings made before implantation of the cells can also be made to provide a baseline measurement. Of course behavioral tests and standard tests for visual acuity can also be performed.

Example 12

Due in part to the unusual appearance of spore-like cells under the light microscope, the cells were examined under an electron microscope. Scanning and electron microscopy was performed according to standard protocols. The electron micrographs revealed several interesting features. For example, the range of spore-like cell sizes may be greater than first appreciated with the light microscope. Some of the spore-like cells shown in Fig. 1A have a diameter of approximately 0.3

microns. The unique cytoarchitecture of the spore-like cell is apparent when viewed with transmission electron microscopy (see Figs. 2A-2D) or following nuclear staining (such as the 4'6-diamidino-2-phenylindole (DAPI) stain described in Example 13). The interior of the spore-like cell is comprised largely of diffuse nuclear material and some cells are surrounded by a "zebra" coating, which is associated with deposits of glycolipids (*i.e.*, carbohydrate and fat). For example, zebra bodies (so-called because of their striped appearance) are associated with mucopolysaccharidoses, such as Hurler's syndrome or with Fabry's disease, in which glycolipids accumulate due to an enzyme deficiency. Spore-like cells thus appear, during at least one stage of their existence, to be unique packets of DNA.

Example 13

A massive accumulation of nuclear material is also apparent when spore-like cells are stained for nucleic acids by methods known to those of ordinary skill in the art. For example, DNA can be stained with either 4'6,-diamidino-2-phenylindole (DAPI) for total DNA staining or with propidium iodide for staining of double-stranded DNA and RNA. DAPI and propidium iodide can be added directly to antifade mounting medium (*e.g.*, 90% glycerol, 1X PBS, and 2.5% 1,4-diazabicyclo[2,2,2]octane (DABCO) (Sigma Chemical Co., St. Louis, MO). Spore-like cells stained with DAPI contained a great deal of nuclear material; the ratio of nuclear to cytoplasmic material was much higher in spore-like cells than one would expect in most fully differentiated cell types.

Example 14

Four tissues (lung, liver, fascia, and spinal cord) were obtained from three animals (Fisher rats) and kept in cold storage for five days. More specifically, each tissue type was removed from an animal less than two hours after the animal was killed and placed in a 50 cc centrifuge tube (Fisher Scientific, Pittsburg, PA) filled with PBS. The tubes were stored at 4°C without supplemental oxygen for five days. Spore-like cells were then isolated as follows.

After excision from the animal, and using sterile technique, the selected tissue was placed in cold PBS containing penicillin (50 mU/ml) and streptomycin (90 mg/ml) (Gibco, Grand Island, NY). The tissue was then manually disassociated with a #11 scalpel, and the disassociated cells were collected by centrifugation at 1200 rpm for five minutes. The tissue was then resuspended in ten ml of 0.05% trypsin (w/v) for five minutes at 37°C. The trypsin was inactivated by adding 10 ml of DMEM/F-12 medium (Gibco) supplemented with 10% heat-inactivated fetal bovine serum (FBS) (Gibco). The cells were then dispersed by trituration using progressively narrower fire-polished, reduced-bore pasteur pipettes. While the aperatures are not measured, the opening of the smallest-bore pipette was approximately 15 μm. The dispersed cells were collected by centrifugation at 1200 rpm for five minutes. The resulting pellet was resuspended in 10 ml of DMEM/F-12 medium containing 33 mM glucose (Sigma Chemical Co., St. Louis, MO), 10 mg/ml transferrin (Sigma), 20 mg/ml insulin (Sigma), 10 mM putrescine (Sigma), 100 nM selenium (Sigma), 10 nM progesterone (Sigma), 20 ng/ml EGF (Peprotech, Rocky Hill, NJ), and 20 ng/ml bFGF (Collaborative Biomedical, Raynham, MA). The primary cell suspension was incubated at 37°C in 5% CO_2, and the media were changed every 3 days. Cells were passaged every 7-9 days by collecting the nonadherent cell aggregates, centrifuging them at 1200 rpm for five minutes and removing the media. Cells were resuspended in fresh media, triturated using narrow fire polished reduced bore pasteur pipettes. The cell suspension was then divided into two suspensions and placed into two new culture dishes.

The technique described above was slightly modified to isolate hepatic tissue: hepatic tissue was washed with cold PBS prior to disassociation.

Standard hematoxalin and eosin (H&E) staining was performed on tissue fixed with 10% formalin. A simple Hall's stain was performed on liver-derived spore-like cells for the presence of bile. Standard stains for mucicarmine and periodic acid-Schiff were also performed.

To assess cellular proliferation, the time that was required for a population of cells to double its number was estimated using periodic phase microscopy field counts (10 fields counted and averaged at 100x) or viable cell counts using Trypan blue with a hemocytometer.

Based on the exclusion of Trypan blue, approximately 50% of the spore-like cells within each of the four tissues that were exposed to 4°C, without supplemental oxygen, for five days, remained viable at the end of that period. Moreover, the spore-like cells isolated from lung, liver, fascia, and spinal cord retained their ability to proliferate and differentiate into tissue-specific structures.

Example 15

After being killed, whole animals (Fisher rats) were placed in plastic bags and stored in a freezer at –86°C. After being frozen for either two or eight weeks, the animals were removed from the freezer and placed in a 37°C water bath until their tissues thawed. Four tissues (lung, liver, fascia, and spinal cord) were then harvested, and spore-like cells were isolated as described in Example 14 and assessed by Trypan blue exclusion for viability. Spore-like cells could be obtained from oxygen-deprived and deeply frozen tissue just as they were from oxygen-deprived and chilled tissue. Approximately 50% of the spore-like cells within each of the four tissues that were exposed to -86°C, without supplemental oxygen, for two or eight weeks, remained viable at the end of those periods. Moreover, the spore-like cells isolated from lung, liver, fascia, and spinal cord retained their ability to proliferate and differentiate into tissue-specific structures.

Example 16

Four tissues (lung, liver, fascia, and spinal cord) were obtained from three animals (Fisher rats) and heated to 85°C for 30 minutes. More specifically, each tissue type was removed from an animal less than two hours after the animal was killed and placed in a 50 cc centrifuge tube (Fisher Scientific, Pittsburg, PA) filled with PBS. The tubes were then placed in a heated water bath as the temperature of the bath was raised to 85°C. The temperature was monitored with sterile thermometers, which were placed within each tube. The tubes were left in the water bath for 45 minutes after the temperature reached 85°C. The tissue was then allowed to cool to room temperature, and spore-like cells were isolated as described in Example 14.

Based on the exclusion of Trypan blue, approximately 50% of the spore-like cells within each of the four tissues that were heated to 85°C for 30 minutes remained viable at the end of that period. Moreover, the spore-like cells isolated from lung, liver, fascia, and spinal cord retained their ability to proliferate and differentiate into tissue-specific structures.

Other Embodiments

One of ordinary skill in the art will appreciate that the spore-like cells described herein can be administered in connection with existing tissue engineering methods, in lieu of differentiated cells in cell-based therapies, and in lieu of cells presently administered following genetic manipulation.

It is to be understood that while the invention has been described in conjunction with the detailed description thereof, that the foregoing description is intended to illustrate and not limit the scope of the invention, which is defined by the scope of the appended claims.

Other aspects, advantages, and modifications are within the scope of the following claims.

What is claimed is:

1. An isolated spore-like cell, wherein the spore-like cell, when first isolated, is:

multipotent;

less than one to approximately seven microns in diameter; and

tolerant of oxygen deprivation.

2. The spore-like cell of claim 1, wherein the cell has one or more areas of high contrast when viewed by transmission electron microscopy.

3. The spore-like cell of claim 2, wherein the areas of high contrast are similar in appearance to the areas of contrast in the spore-like cell shown in Figs. 2C and 2D.

4. An isolated spore-like cell, wherein the spore-like cell remains viable following exposure for more than ten minutes to an environment that is at least 42°C or less than 0°C.

5. The spore-like cell of claim 4, wherein the cell has one or more areas of high contrast when viewed by transmission electron microscopy.

6. The spore-like cell of claim 5, wherein the areas of high contrast are similar in appearance to the areas of contrast in the spore-like cell shown in Figs. 2C and 2D.

7. The spore-like cell of any of claims 3 to 6, wherein the cell is less than one to approximately seven microns in diameter.

8. The spore-like cell of any of claims 1 to 7, wherein the cell is isolated from a post-natal animal.

9. The spore-like cell of claim 8, wherein the post-natal animal is an adult animal.

10. The spore-like cell of claim 8, wherein the post-natal animal is a post-natal human.

11. The spore-like cell of claim 10, wherein the post-natal human is an adult human.

12. The spore-like cell of any of claims 1 to 7, wherein the cell is isolated from a deceased animal.

13. The spore-like cell of claim 12, wherein the deceased animal is a deceased human.

14. The spore-like cell of any of claims 1 to 7, wherein the cell is isolated from a tissue that develops from the endoderm.

15. The spore-like cell of any of claims 1 to 7, wherein the cell is isolated from a tissue that develops from the mesoderm.

16. The spore-like cell of any of claims 1 to 7, wherein the cell is isolated from a tissue that develops from the ectoderm.

17. The spore-like cell of any of claims 1 to 7, wherein the cell is approximately one-tenth to one-third of a micron in diameter.

18. The spore-like cell of any of claims 1 to 7, wherein the cell is approximately one micron in diameter.

19. The spore-like cell of any of claims 1 to 7, wherein the cell is approximately one to three microns in diameter.

20. The spore-like cell of any of claims 1 to 7, wherein at least about half the volume of the cell is comprised of nucleic acids.

21. The spore-like cell of any of claims 1 to 7, wherein, when first isolated, the cell fails to express the protein nestin when analyzed by immunocytochemistry.

22. The spore-like cell of any of claims 1 to 7, wherein the cell, when isolated from a post-natal mammal and placed in a damaged, infected, or malfunctioning tissue, develops into a cell having a phenotype substantially similar to the phenotype of a healthy cell normally found in the tissue.

23. The spore-like cell of claim 22, wherein the cell, when isolated from the dermis of a post-natal mammal and placed in a dermal wound, develops into a cell having a phenotype substantially similar to that of a sympathetic or parasympathetic neuron, or a cell within a sweat gland, a sebaceous gland, or a hair follicle.

24. The spore-like cell of claim 22, wherein the cell, when isolated from the epidermis of a post-natal mammal and placed in an epidermal wound, develops into a cell having a phenotype substantially similar to that of a melanocyte, a keratinocyte, or a Merkel cell.

25. The spore-like cell of claim 22, wherein the cell, when isolated from the retina of a post-natal mammal and placed in a damaged or malfunctioning retina, develops into a cell having a phenotype substantially similar to that of a pigmented epithelial cell, a photoreceptor cell, a bipolar cell, a horizontal cell, an amacrine cell, a ganglion cell, an interplexiform cell, or a radial cell of Muller.

26. The spore-like cell of claim 22, wherein the cell, when isolated from the pancreas of a post-natal mammal and placed in a damaged or malfunctioning pancreas, develops into a cell that produces glucagon, somatostatin, pancreatic polypeptide, or insulin.

27. The spore-like cell of claim 22, wherein the cell, when isolated from the lung of a post-natal mammal and placed in a damaged or malfunctioning lung, develops into a cell that exchanges oxygen or secretes a surfactant.

28. A tissue construct comprising the cell of any of claims 1 to 27.

29. The construct of claim 28, further comprising a support structure.

30. The construct of claim 29, further comprising hydrogel, wherein the hydrogel and the cell of any of claims 1 to 27 form a hydrogel-spore-like cell composition.

31. A method for isolating a spore-like cell, the method comprising dissociating a tissue sample and passing the dissociated tissue sample through a first device having an aperture no greater than about 15 microns.

32. The method of claim 31, further comprising passing the tissue sample through a second device having an aperture greater than 15 microns before the sample is passed through the first device.

33. A method for isolating a spore-like cell, the method comprising passing a tissue sample through a series of devices having progressively smaller apertures, the smallest aperture being about 15 microns.

34. The method of any of claims 31 to 33, wherein the first device, the second device, or one or more of the series of devices is a pipette.

35. The method of any of claims 31 to 33, wherein the first device, the second device, or one or more of the series of devices is a filter.

36. The method of any of claims 31 to 33, further comprising exposing the tissue sample to a digestive enzyme prior to passage through the first device, the second device, or one or more of the series of devices.

37. The method of claim 36, wherein the digestive enzyme is trypsin or collagenase.

38. The method of claim 37, wherein the tissue is incubated with approximately 0.05% trypsin at 37°C for approximately five minutes.

39. A method for isolating a spore-like cell from a biological sample, the method comprising exposing the sample to an oxygen-deficient environment for a time sufficient to kill substantially all of the non-spore-like cells in the sample and culturing the sample, thereby isolating spore-like cells from the sample.

40. A method for isolating a spore-like cell from a biological sample, the method comprising exposing the sample to a non-physiological temperature for a time sufficient to kill substantially all of the non-spore-like cells in the sample and culturing the sample, thereby isolating spore-like cells from the sample.

41. A method for generating an artificial tissue, the method comprising combining hydrogel with the spore-like cell of any of claims 1 to 27.

42. The method of claim 41, further comprising delivering the combined hydrogel and cell into a permeable, biocompatible support structure.

43. Use of the spore-like cell of any of claims 1 to 27 for treating a patient who has cancer, the cell being applied to an area within the patient from which a tumor has been removed or, where the cancer is a cancer of a blood-borne cell, to the bloodstream.

44. Use of the spore-like cell of any of claims 1 to 27 for treating a patient who has a damaged, infected, or malfunctioning tissue, the cell being applied to the damaged, infected, or malfunctioning tissue.

45. Use of the spore-like cell according to claim 44, wherein the patient has a skin wound, a degenerative disease, a pulmonary disease, a liver disease, or diabetes.

46. Use of the spore-like cell of any of claims 1 to 27 for the manufacture of a medicament for treating a patient who has cancer, the medicament being suitable for application to an area within the patient from which a tumor has been removed or, where the cancer is a cancer of a blood-borne cell, to the bloodstream.

47. Use of the spore-like cell of any of claims 1 to 27 for the manufacture of a medicament for treating a patient who has a damaged, infected, or malfunctioning tissue, the medicament being suitable for application to the damaged, infected, or malfunctioning tissue.

48. Use of the spore-like cell according to claim 47, wherein the patient has a skin wound, a degenerative disease, a pulmonary disease, a liver disease, or diabetes.

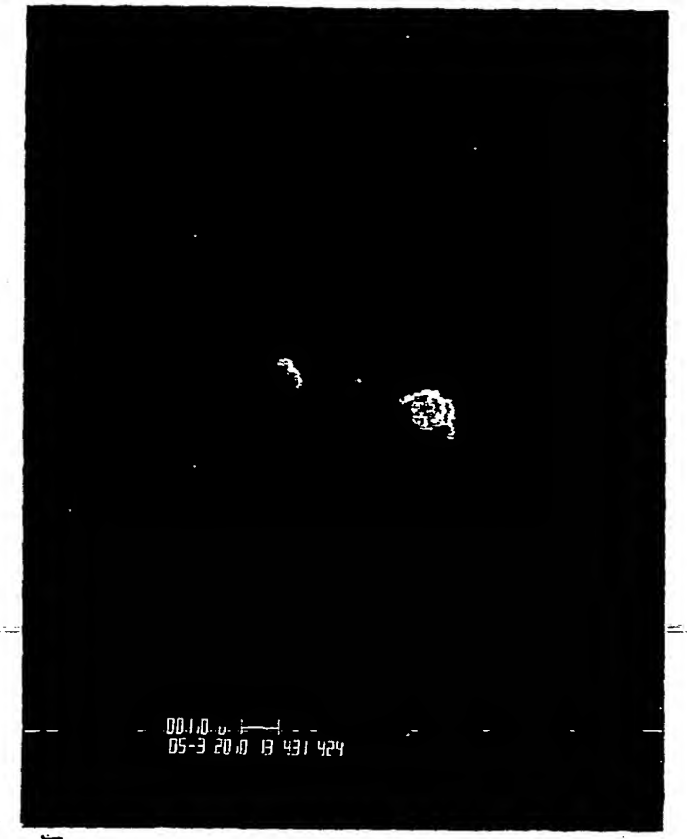

Fig 1A

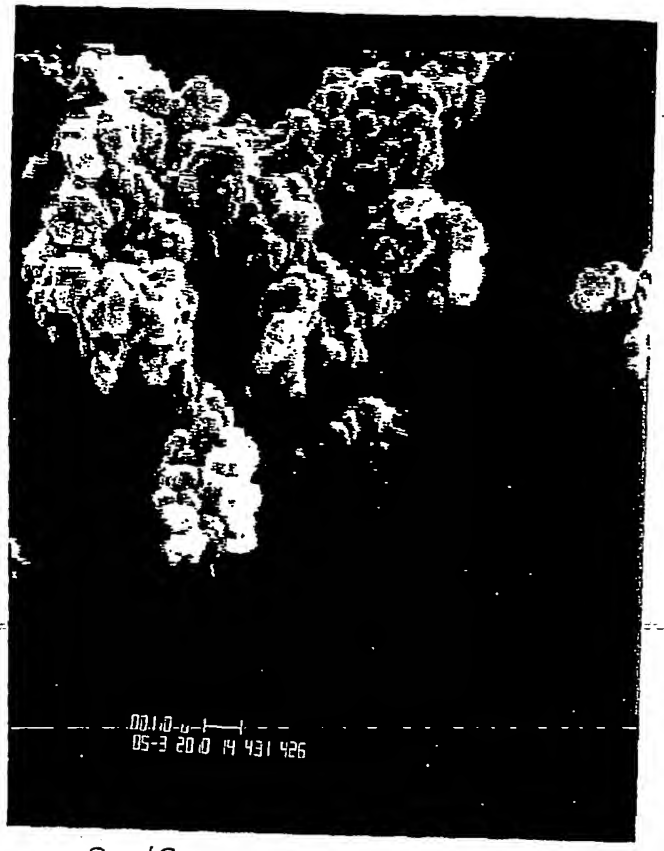

Fig 1B

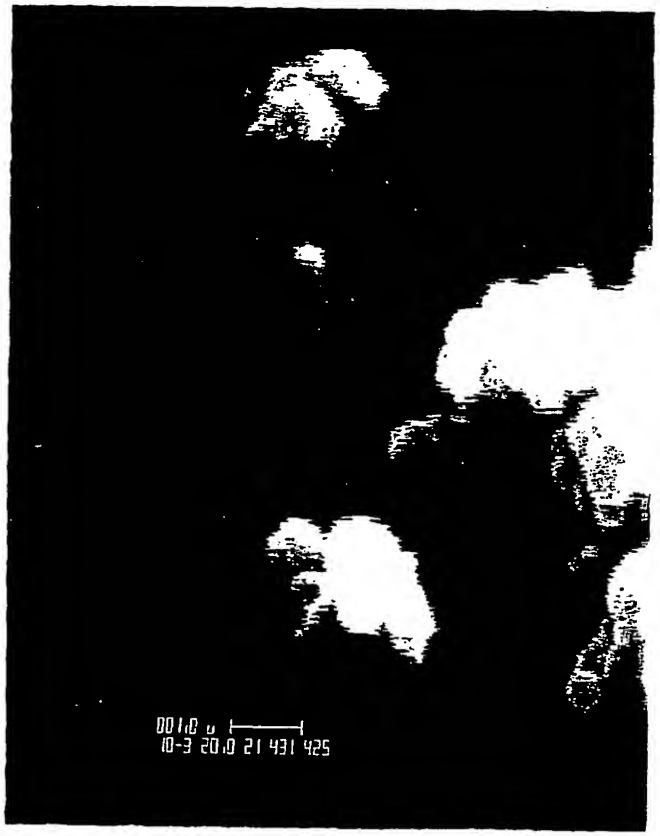

FIG. 1C

Fig 2A

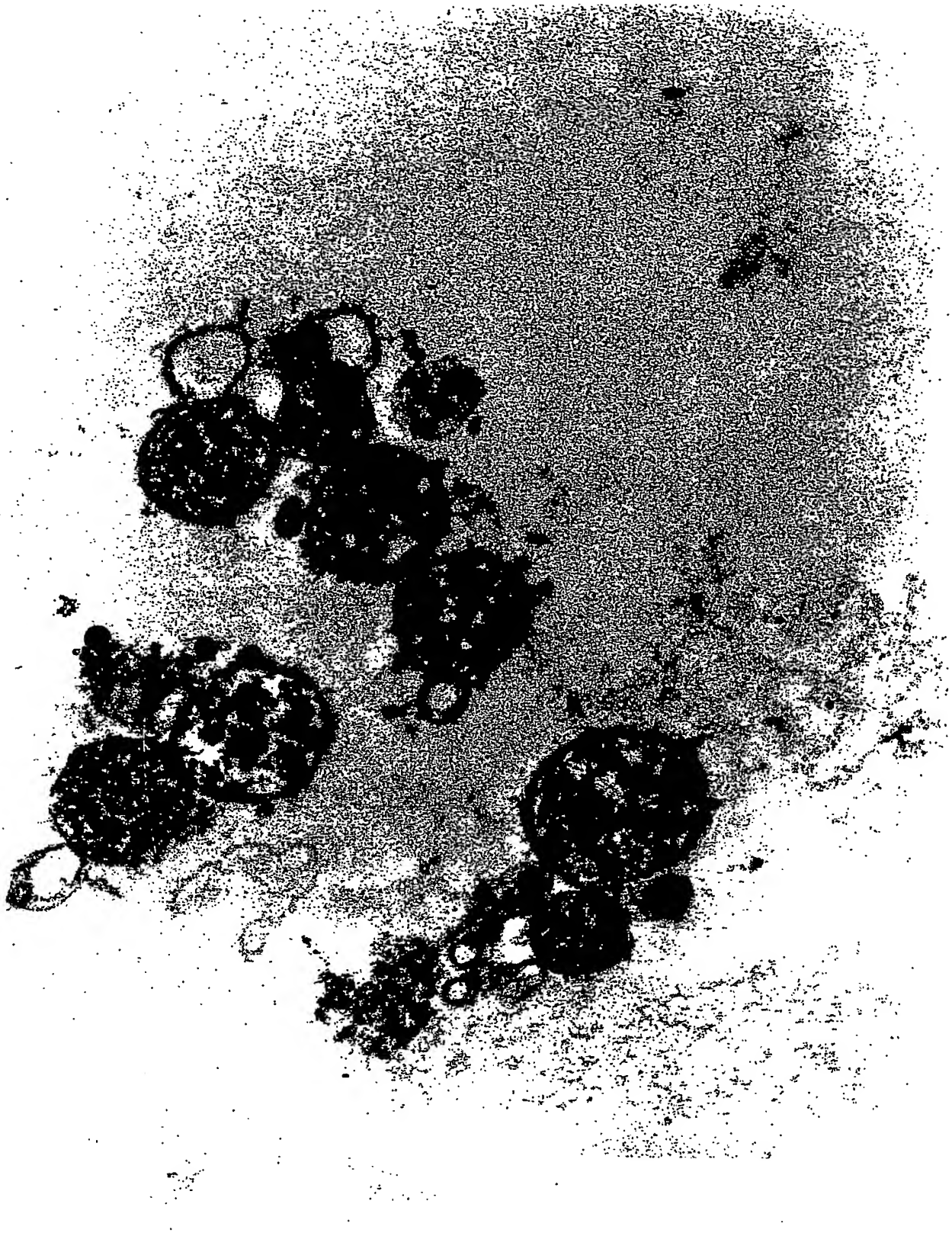

Fig 2B

Fig 2C

Fig 2D

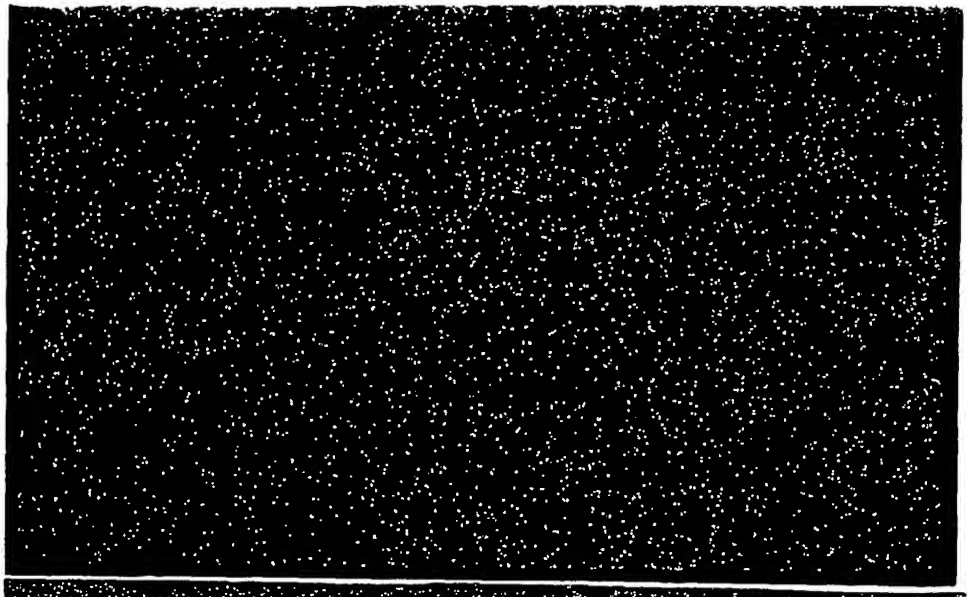

Fig. 3A

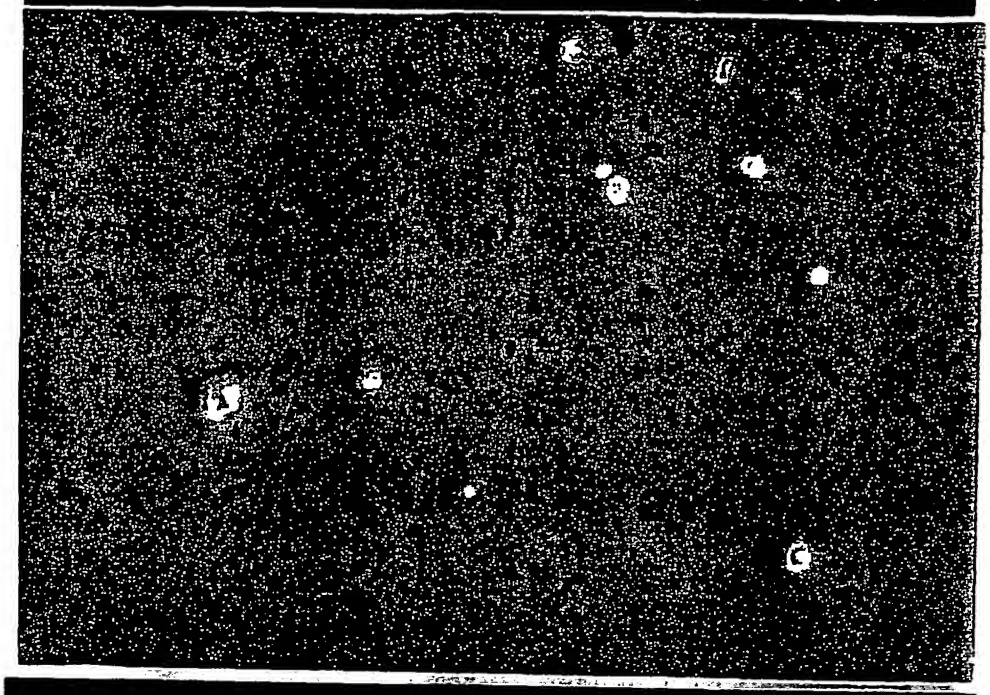

Fig. 3B

Fig. 3C

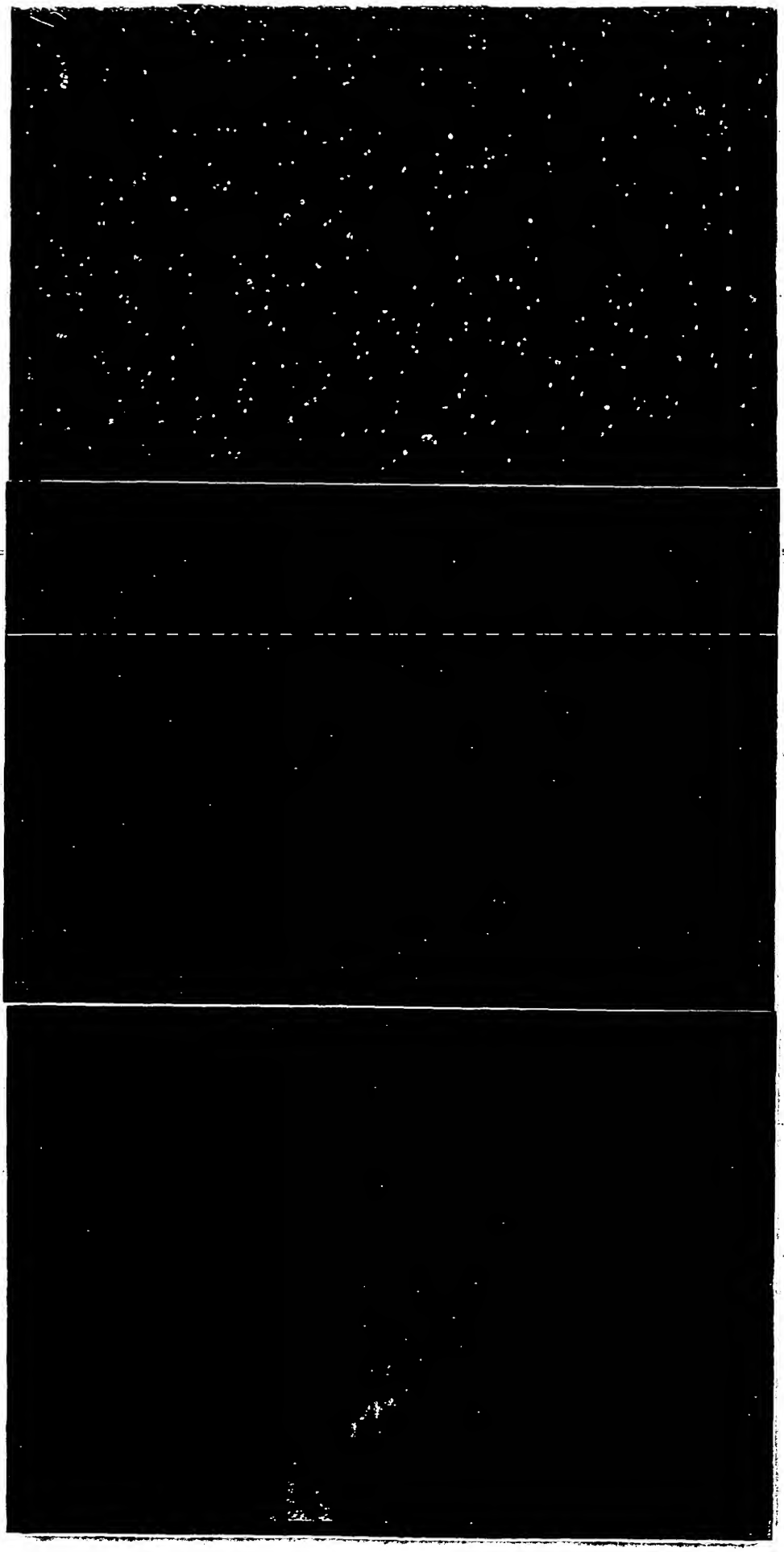

Fig. 4A

Fig. 4B

Fig. 4C

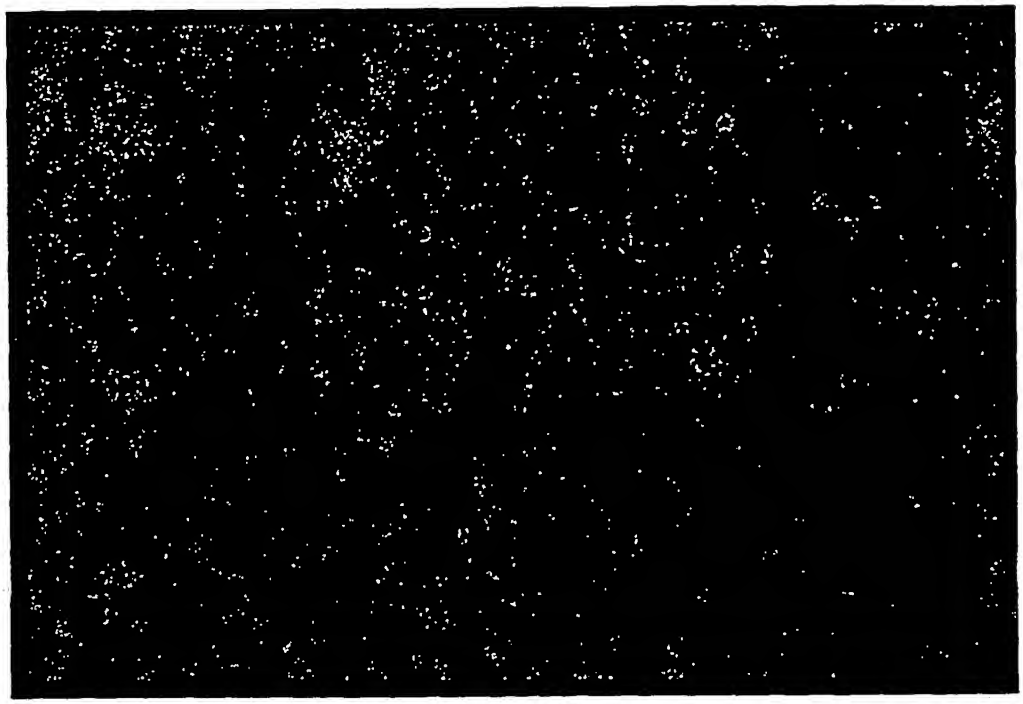

Fig. 5A

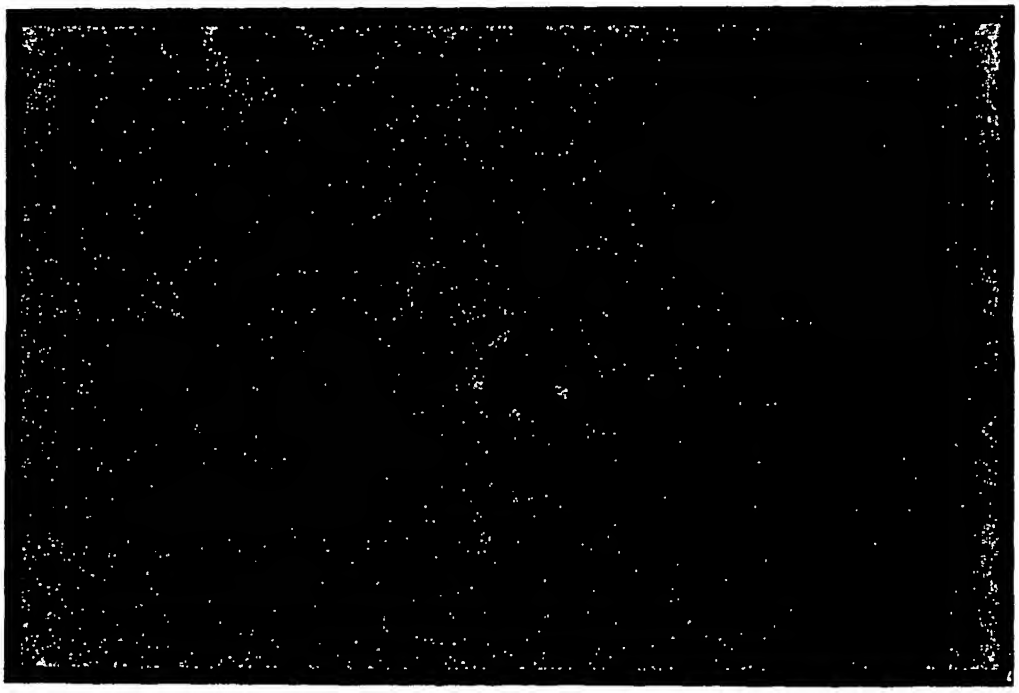

Fig. 5B

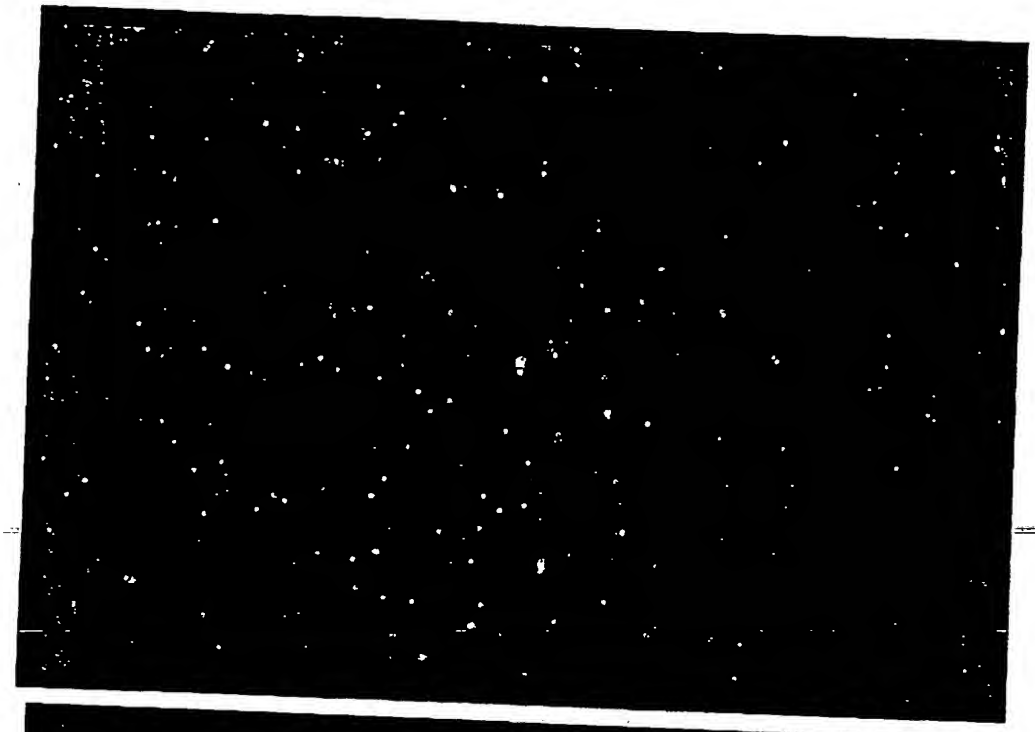

Fig. 6A

Fig. 6B

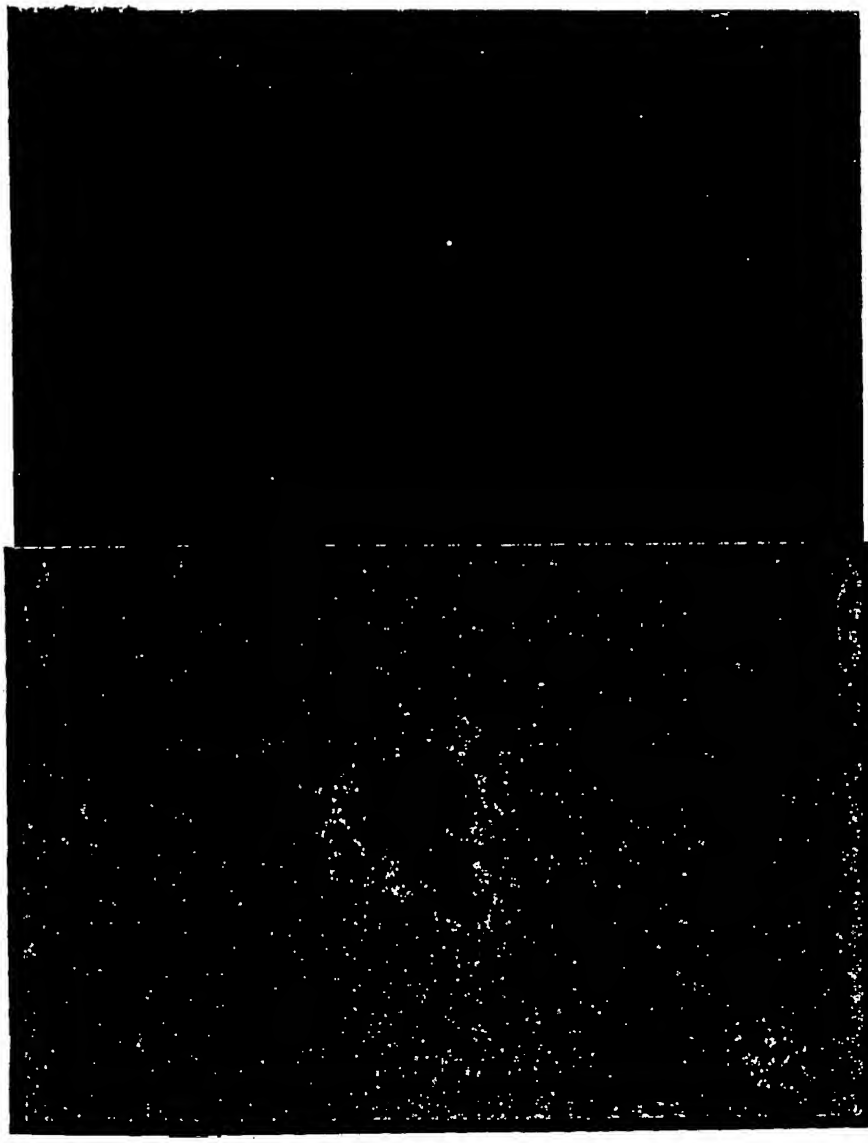

Fig. 7A

Fig. 7B

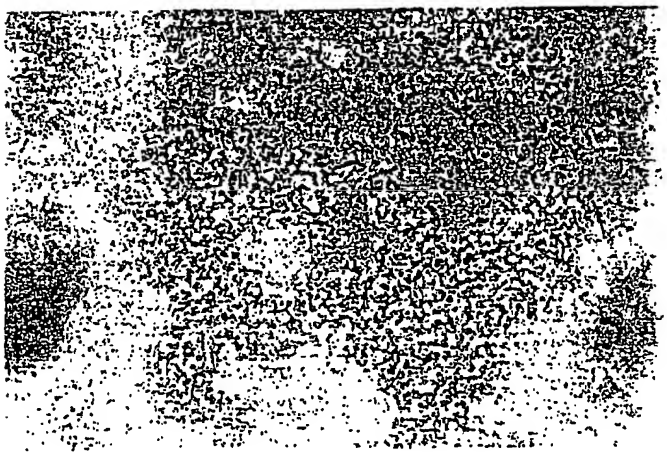

Fig. 7C

Fig. 7D

Fig. 7E

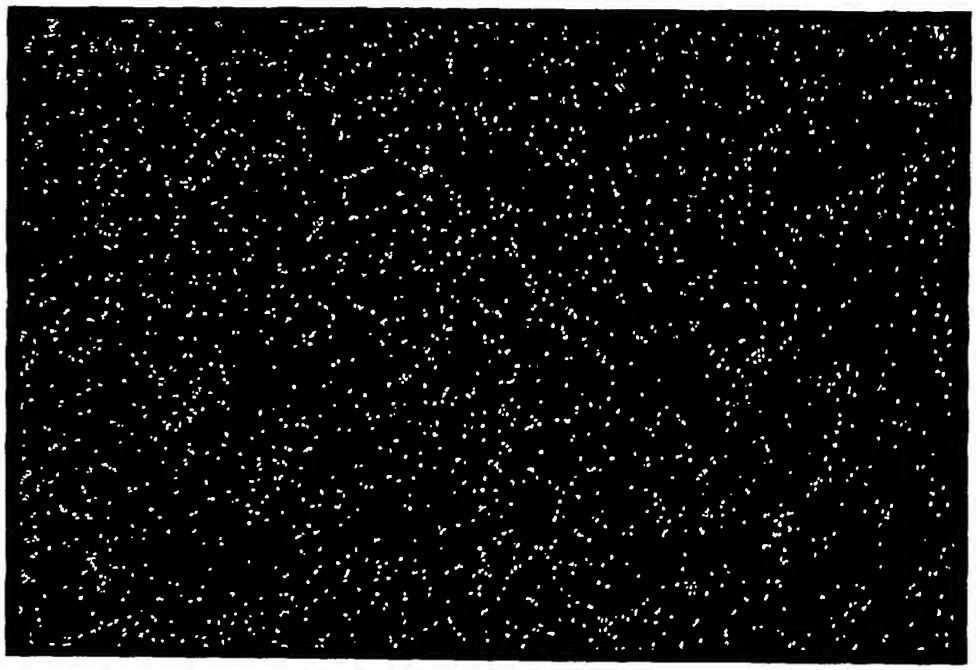

Fig. 8A

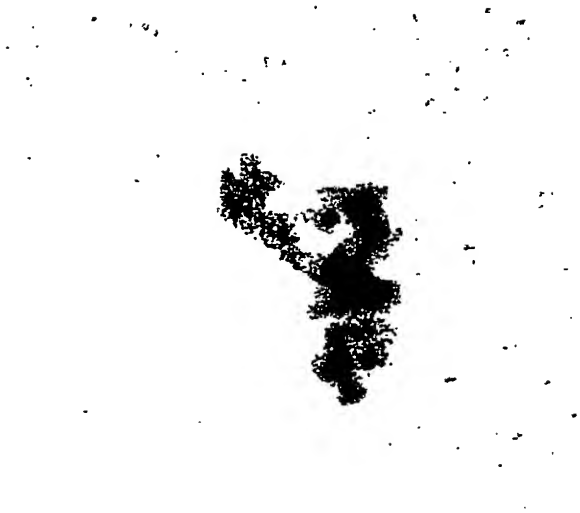

Fig. 8B

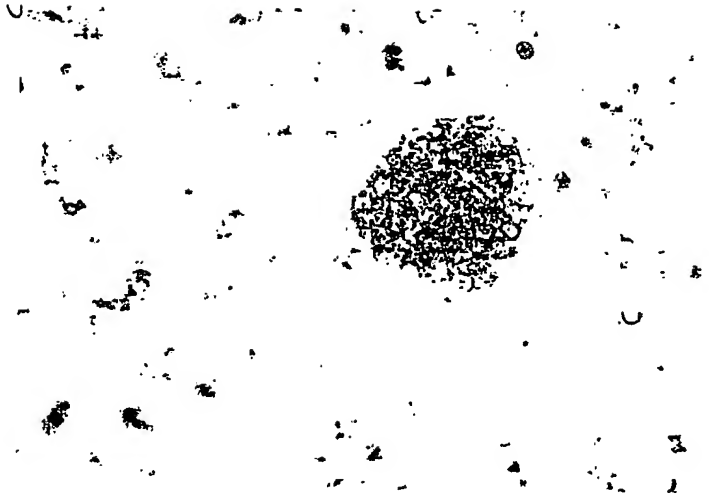

Fig. 8C

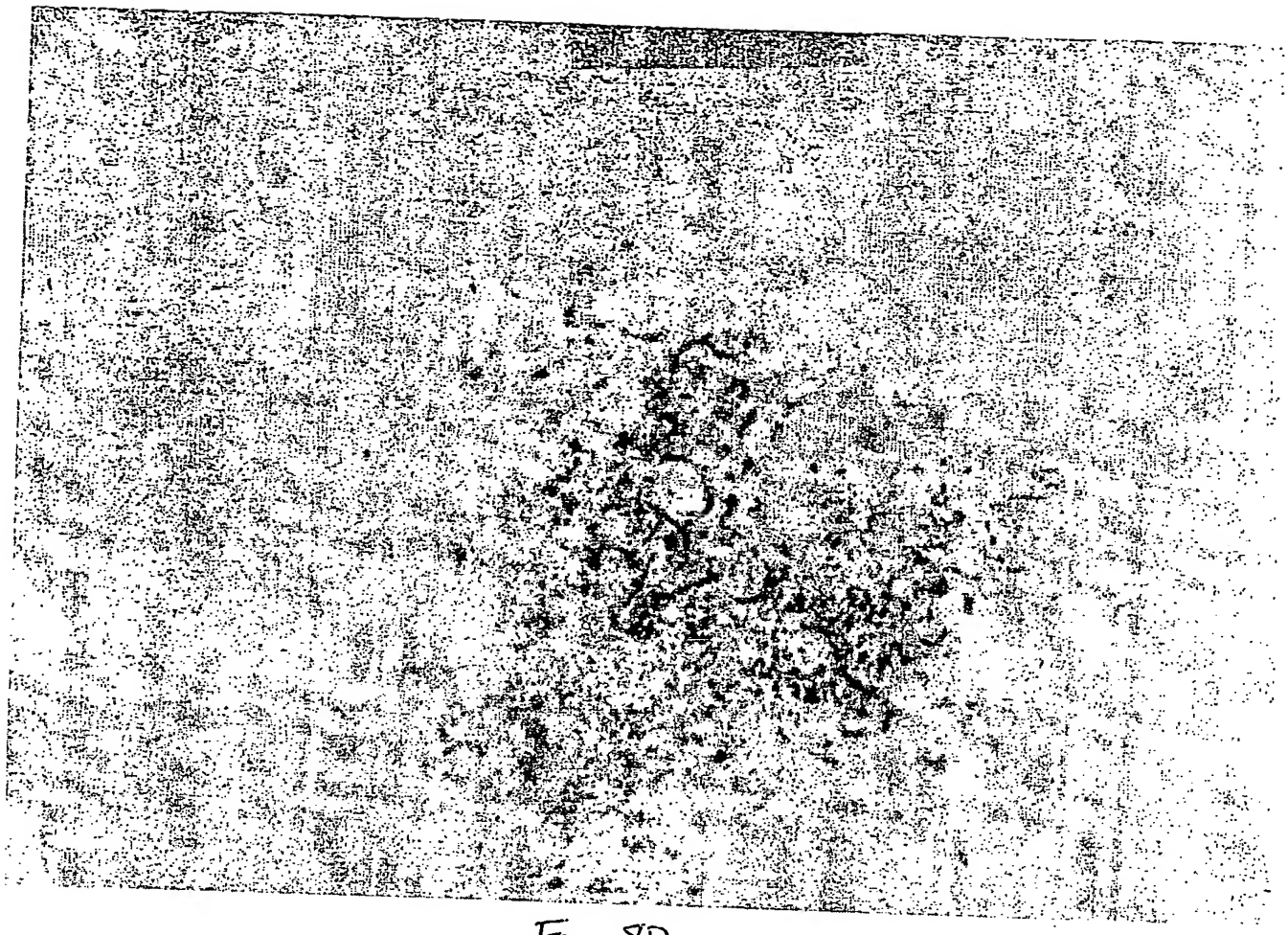

Fig. 8D

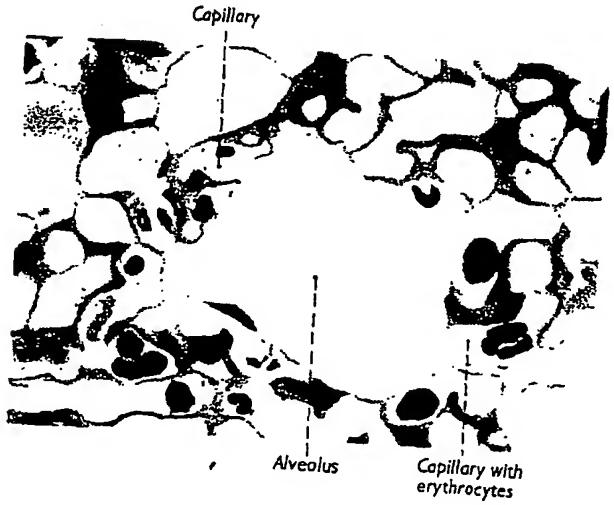

Fig. 8E

Fig 9A

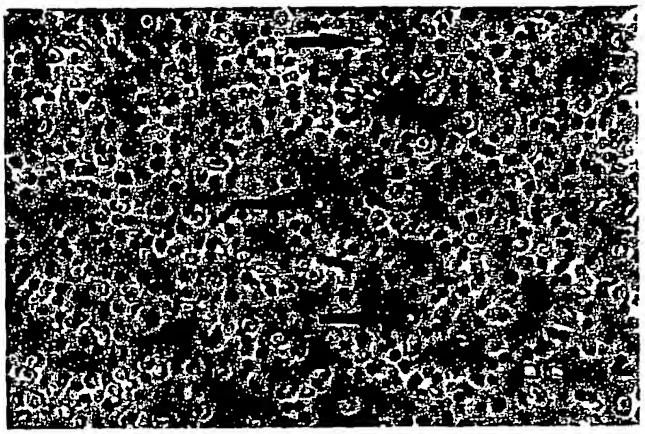

Fig. 9B

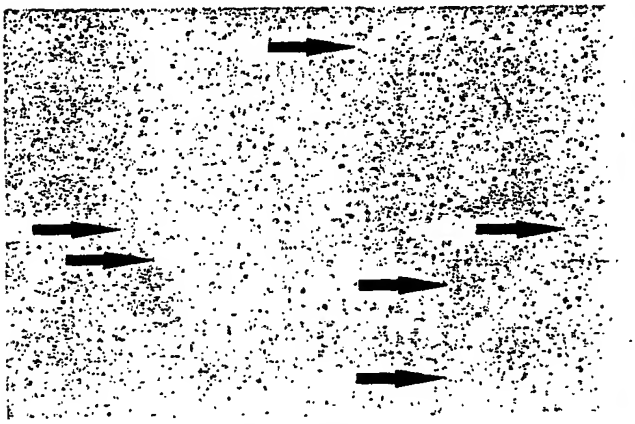

Fig 9C

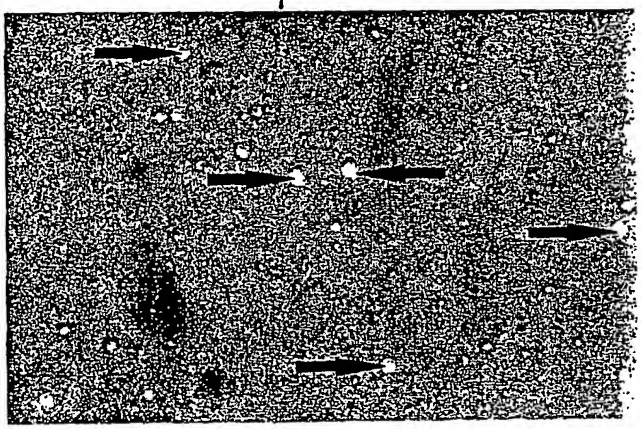

Fig. 9D

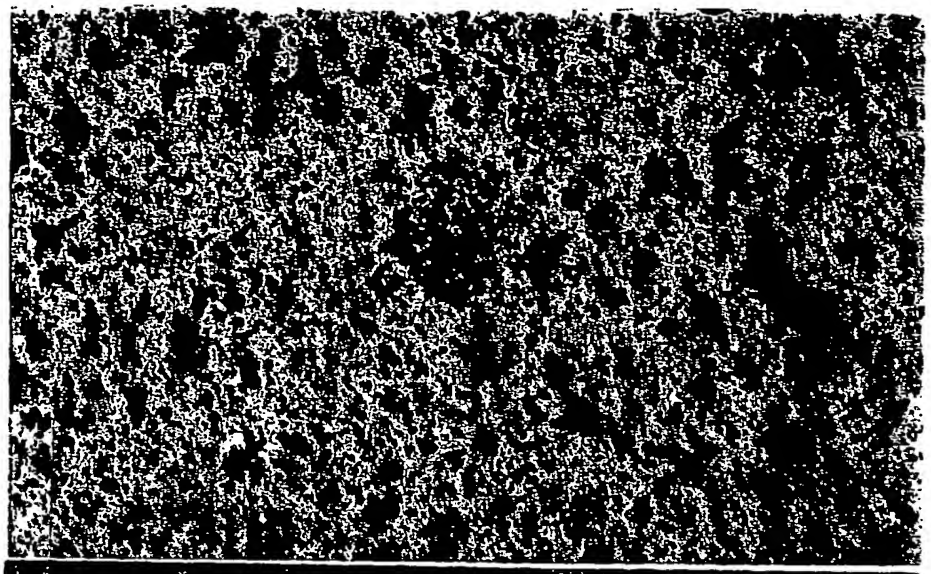

Fig. 10A

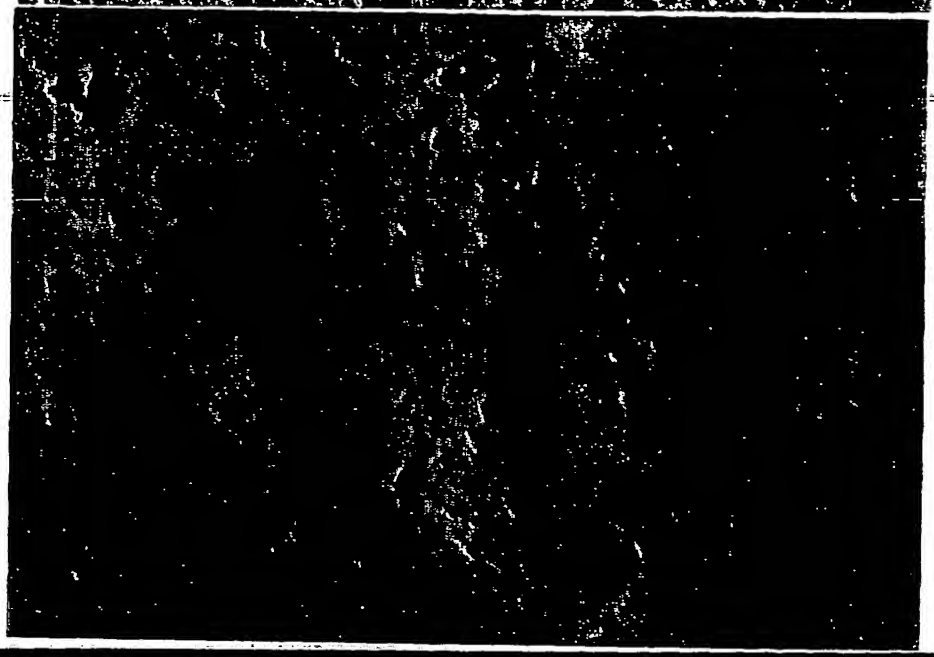

Fig. 10B

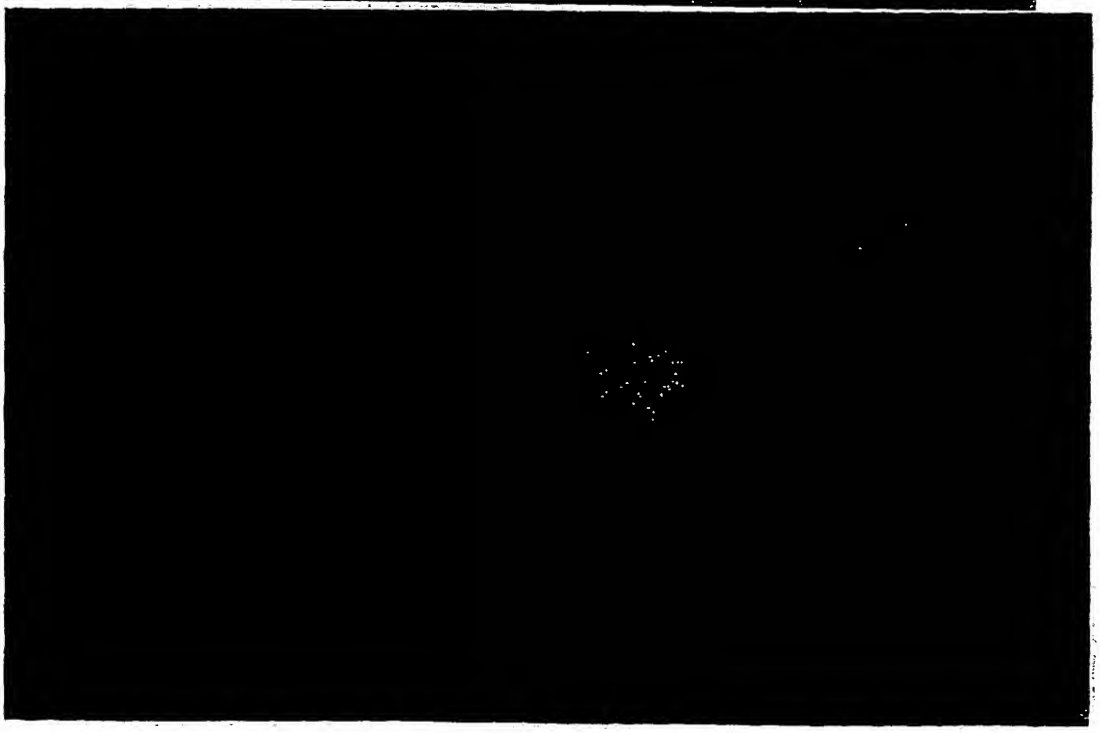

Fig. 10C

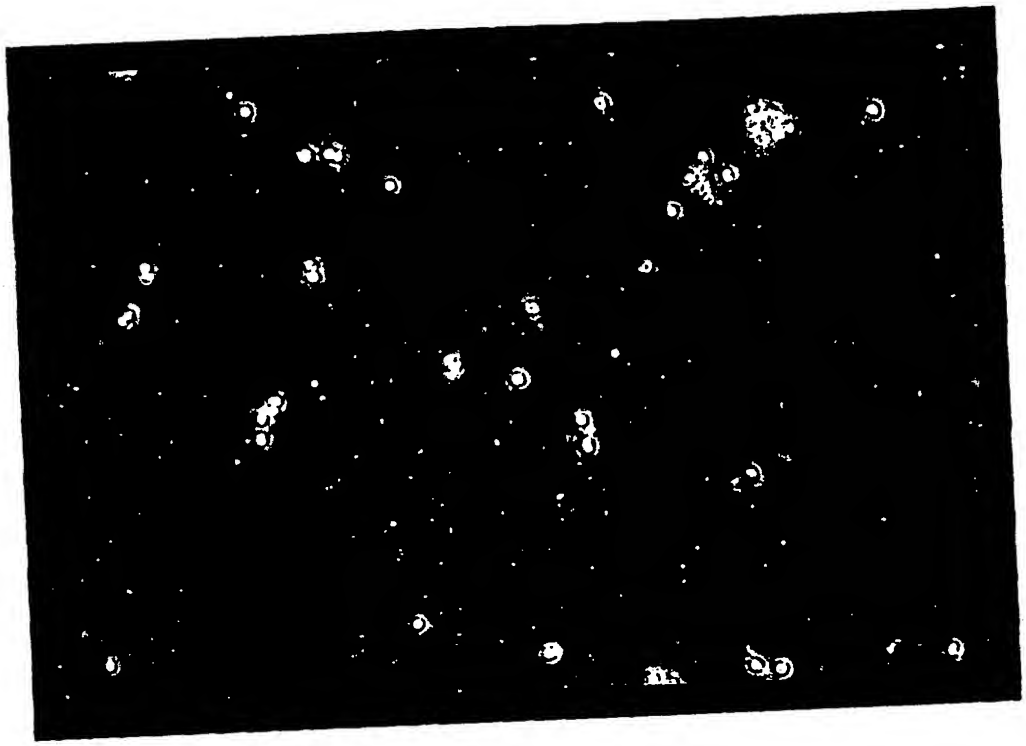

Fig. 11

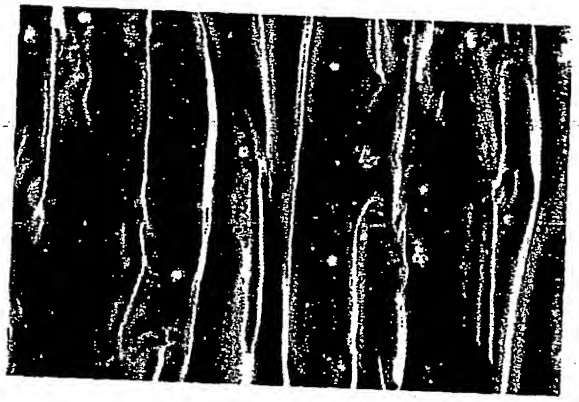

Fig. 12A

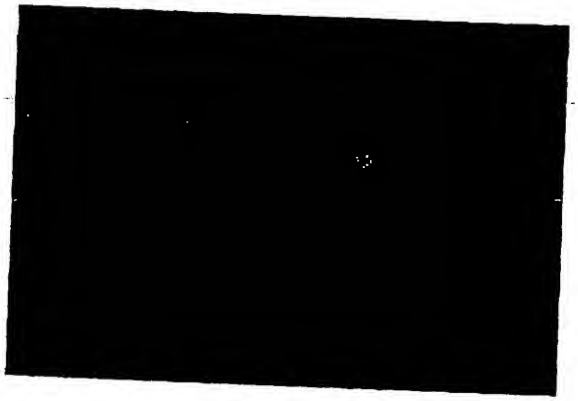

Fig. 12B

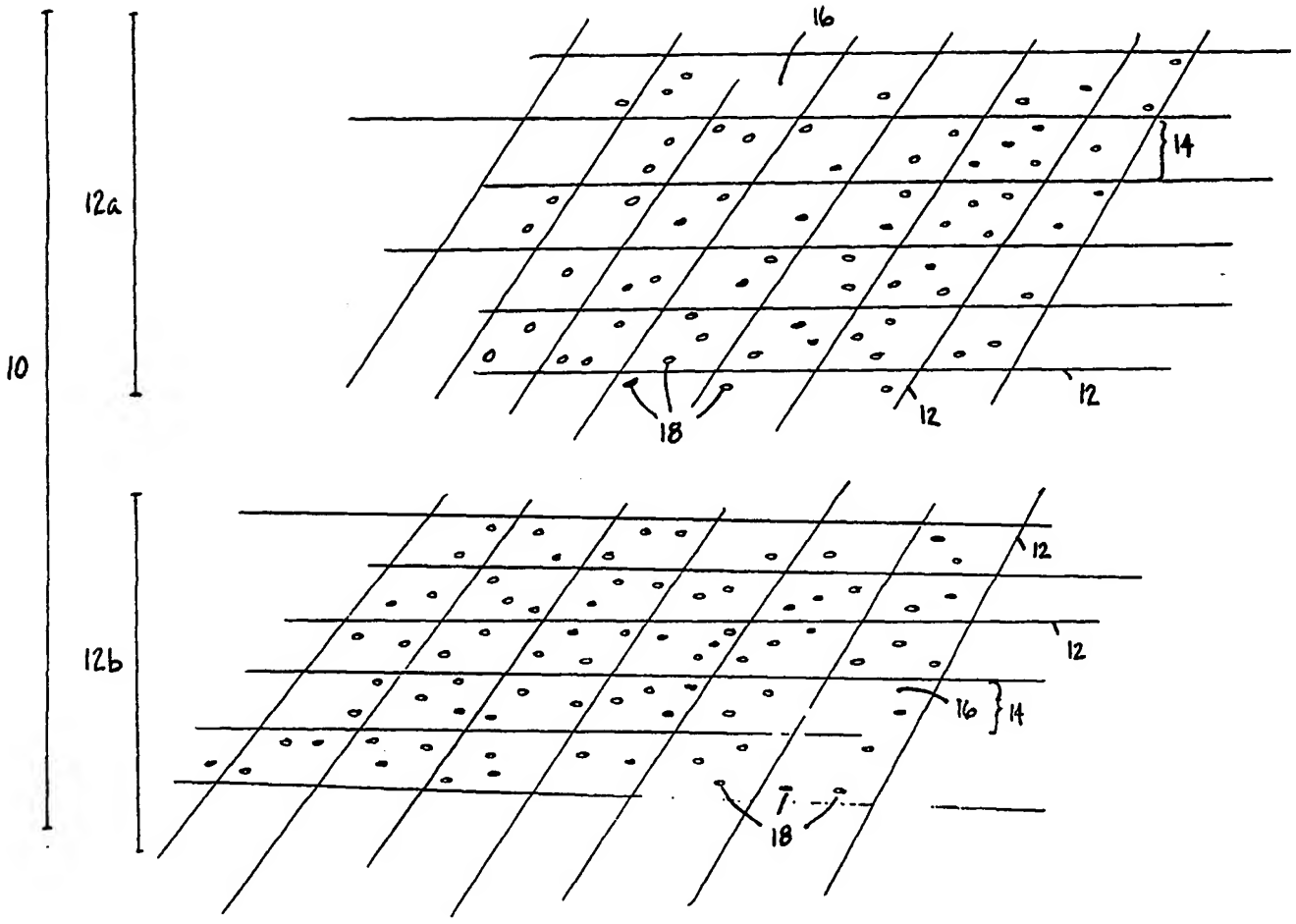

Fig. 13

INTERNATIONAL SEARCH REPORT

International application No.
PCT/US00/35606

A. CLASSIFICATION OF SUBJECT MATTER
IPC(7) : A01N 1/00, 1/02, 63/00; C12N 5/00
US CL : 435/1.1, 325; 424/93.1
According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)
U.S. : 435/1.1, 325; 424/93.1

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practicable, search terms used)
MEDLINE, STN, EAST

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
Y	TEITELMAN, G. et al. Precursor cells of mouse endocrine pancras coexpress insulin, glucagon and the neuronal proteins tyrosine hydroxylase and neuropeptide Y, but not pancreatic polypeptide. Development. 1993. Vol 118. pages 1031-1039, especially figures.	1-6
Y	CORNELIUS, J.G. et al. In vitro-generation of isltes in long-term cultures of pluripotent stem cells from adult mouse pancreas. Horm. Metab. Res 1997. Vol 29. pages 271-277, especially discussion section.	1-6, 39-40

[X] Further documents are listed in the continuation of Box C. [ ] See patent family annex.

* Special categories of cited documents:

"A" document defining the general state of the art which is not considered to be of particular relevance

"E" earlier document published on or after the international filing date

"L" document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)

"O" document referring to an oral disclosure, use, exhibition or other means

"P" document published prior to the international filing date but later than the priority date claimed

"T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention

"X" document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone

"Y" document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art

"&" document member of the same patent family

Date of the actual completion of the international search
01 MARCH 2001

Date of mailing of the international search report
26 APR 2001

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C (Continuation). DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
Y	GAGE, F.H. et al. Survival and differentiation of adult neuronal progenitor cells transplnated to the adult brain. Proc. Natl. Acad. Sci. December 1995. Vol 92. pages 11879-11883, entire document.	1-6, 39-40
Y	MOLLER, C.J. et al. Differential expression of neural cell adhesion molecule and cadherins in pancreatic islets, glucagomas, *and insulinomas. Molecular endocrinology 1992. Vol6. No. 8.* pages 1332-1342, entire document-especially figures.	1-6
Y	CRAIG, C.G. et al. In vivo growth factor expansion of endognous subepedymal neural precusor cell populations in the adult mouse brain. The journal of neuroxcience 15 April 1996. Vol 16. No. 8. pages 2649-2658, especially introduction and discussion sections.	1-6, 39-40

INTERNATIONAL SEARCH REPORT

International application No.
PCT/US00/35606

Box I Observations where certain claims were found unsearchable (Continuation of item 1 of first sheet)

This international report has not been established in respect of certain claims under Article 17(2)(a) for the following reasons:

1. ☐ Claims Nos.:
because they relate to subject matter not required to be searched by this Authority, namely:

2. ☐ Claims Nos.:
because they relate to parts of the international application that do not comply with the prescribed requirements to such an extent that no meaningful international search can be carried out, specifically:

3. ☒ Claims Nos.: 7-38 and 41-48
because they are dependent claims and are not drafted in accordance with the second and third sentences of Rule 6.4(a).

Box II Observations where unity of invention is lacking (Continuation of item 2 of first sheet)

This International Searching Authority found multiple inventions in this international application, as follows:

Please See Extra Sheet.

1. ☐ As all required additional search fees were timely paid by the applicant, this international search report covers all searchable claims.

2. ☒ As all searchable claims could be searched without effort justifying an additional fee, this Authority did not invite payment of any additional fee.

3. ☐ As only some of the required additional search fees were timely paid by the applicant, this international search report covers only those claims for which fees were paid, specifically claims Nos.:

4. ☐ No required additional search fees were timely paid by the applicant. Consequently, this international search report is restricted to the invention first mentioned in the claims; it is covered by claims Nos.:

Remark on Protest

☐ The additional search fees were accompanied by the applicant's protest.

☐ No protest accompanied the payment of additional search fees.

BOX II. OBSERVATIONS WHERE UNITY OF INVENTION WAS LACKING
This ISA found multiple inventions as follows:

This application contains the following inventions or groups of inventions which are not so linked as to form a single inventive concept under PCT Rule 13.1.

Group I, claim(s)1-6, drawn to an isolated spore-like cell.
Group II, claim(s) 39-40, drawn to a method of isolating a spore-like cell.

The inventions listed as Groups I and II do not relate to a single inventive concept under PCT Rule 13.1 because, under PCT Rule 13.2, they lack the same or corresponding special technical features for the following reasons: An isolated cell resembling a spore-like cell, and isolated by the same method steps as described in the Examples of the present specification has previously been described in art, for example in Cornelius et al. and Teitelman et al. Further, attempts to use isolated cells for treatment or reconstruction of a tissue/organ have been also described in the art, for example Gage et al. Therefore, the claims as written encompassing the isolated cells and methods of use do not contribute a special technical feature which was not previously described in the art.